Immunological Study of Responses to a Meningococcal Antigen Using a Multiplex Antibody Binding Assay

Thesis

Immunological Study of Responses to a Meningococcal Antigen Using a Multiplex Antibody Binding Assay

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Declaration

I declare that the work presented in this thesis is my own personal effort. Where any of the content presented is the result of input or data from others, this is duly acknowledged in the text. To that effect, it must be noted that P1._,_ mutant of PorA was previously cloned by Dr. Hannah Chan, who has also used the PorA and FetA proteins for immunisation work confirming the correct epitope presentation of these antigens. CD analysis on the purified proteins was carried out with these proteins with the help of Dr. Angela Martina, however work is not presented in this thesis. Reasonable care was taken to ensure the work presented was original, and to the best of my knowledge does not breach copyright law. Where work was taken from outsides sources, these were cited and acknowledged within the text.

Abstract

Neisseria meningitidis is an important human pathogen and a major cause of septicaemia and meningitis worldwide. The immunodominant antigen of *N. meningitidis* is the highly variable OMP, PorA and detection of serum anti-PorA antibodies (IgG) is classically performed through use of the enzyme-linked immunosorbent assay (ELISA). Whilst sensitive and reliable, these methods are labour and reagent intensive particularly for detection of multiple anti-PorA antibodies within a single serum sample.

This thesis describes the development and evaluation of an assay for simultaneous detection of antibodies directed to eight serosubtypes of PorA. A panel of purified meningococcal proteins were developed including seven serosubtypes of PorA along with a variable regions 1 and 2 deleted mutant; three serotypes of PorB and five variants of FetA. PorA proteins were conjugated to fluorescent microsphere sets and assessed using serum from a range of sources including preclinical and clinical trials and carriage and seroepidemiology studies.

No evidence of microsphere interference was observed between monoplex and multiplex assays over a range of dilutions. The multiplex assay was specific; sensitive, with low limits of detection (≤176.88 pg/ml); and reproducible for the measurement of serotype specific anti-PorA antibodies, with low intra- and inter-assay variability.

Increases in serum IgG against specific PorA serosubtypes were detected using the assay in serum from a range of sources. However with the complexity of adult serum, it is difficult to distinguish between pre-existing and induced responses and may result in what appear to be non-specific responses. Lack of a suitable human standard has hindered the quantification of antibodies and an arbitrary concentration was assigned for each PorA serosubtype. However, advantages of small sera volumes, high throughput of sera, simplicity of the assay, and the ability to extend the assay, make the PorA multiplex assay a viable alternative to the standard ELISA.

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List of Abbreviations

4PL ..... 4 Parameter Logistic regression curve

AFLP ..... Amplified Fragment Length Polymorphism

APS ..... Ammomium Persulphate

$BIGS_{DB}$ ..... Bacterial Isolate Genome Sequence Database

BSA ..... Bovine Serum Albumin

CI ..... Confidence Interval

CV ..... Coefficient of Variation

dATP ..... 2'-Deoxyadenosine-5'-Triphosphate

DNA ..... Deoxyribonucleic Acid

DTT ..... Dithiothreitol

EDC ..... 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA ..... Ethylenediaminetetra-acetic Acid

ELISA ..... Enzyme-Linked Immunosorbent Assay

EMLST ..... Extended Multilocus Sequence Typing

ET ..... Electrophoretic Types

fHbp ..... Factor H binding protein

gDNA ..... Genomic DNA

GMT ..... Geometric Mean Titre

HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hib ..... *Haemophilus influenzae type* b

HPA ..... Health Protection Agency

HCl ..... Hydrochloric Acid

IgG ..... Immunoglobin G

IPTG ..... Isopropyl β-D-1-thiogalactopyranoside

Kan ..... Kanamycin

KCl ..... Potassium Chloride

kDa ..... Kilo Dalton

LB ..... Luria Broth

LBKan ..... Luria Broth supplemented with 30 µg/ml kanamycin

LIC ..... Ligation Independant Cloning

LPS ..... Lipopolysaccharide

MAb ..... Monoclonal Antibody

MATS ..... Meningococcal Antigen Typing System

MCC ..... Meningococcal serogroup C Conjugate vaccine

MES ..... 2-(N-morpholino)ethanesulfonic acid

MFI ..... Mean Fluorescence Intensity

MLEE ..... Multilocus Enzyme Electrophoresis

MLST ..... Multilocus Sequence Typing

N. meningitidis ..... *Neisseria meningitidis*

NaCl ..... Sodium Chloride

NadA ..... Neisserial adhesion A

NaOH ..... Sodium Hydroxide

N/D ..... Not Determined

NHBA ..... Neisserial Heparin-Binding Antigen

NHS ..... N-hydroxysuccinimide

NIPH ..... Norwegian Institute of Public Health

Ni-NTA ..... Nickel-Nitriloacetic Acid

Nm ..... *Neisseria meningitidis* isolate

NOMV ..... Native Outer Membrane Vesicle

NT ..... No serotyping information available

NTA ..... N-(5-amino-1-carboxypentyl)iminodiacetic acid

NVEC ..... National Vaccine Evaluation Consortium

OD_{600} ..... Optical Density Wavelength of 600nm

OMP ..... Outer Membrane Protein

OMV ..... Outer Membrane Vesicle

P1._,_ ..... VR1 and VR2 deleted mutant of PorA

PAGE ..... Polyacrylamide Gel Electrophoresis

PBS ..... Phosphate Buffered Saline

PCR ..... Polymerase Chain Reaction

PEG ..... Poly Ethylene Glycol

PFGE ..... Pulsed-Field Gel Electrophoresis

PL ..... Phospholipid

RIVM ..... National Institute for Public Health and the Environment, NL

RMLST ..... Ribosomal Multilocus Sequence Typing

Rmp ..... Reduction Modifiable Protein

RNA ..... Ribonucleic Acid

rPE ..... Recombinant Phycoerythrin

SBA ..... Serum Bactericidal Antibody

SD ..... Standard Deviation

SDS ..... Sodium Dodecyl Sulphate

SEU ..... Seroepidemiology Unit

SOC ..... Super Optimal Broth

ST ..... Sequence Type

TBS ..... Tris Buffered Saline

TEMED ..... Tetramethylethylenediamine

UK-MPHLS ..... UK-Manchester Public Health Laboratory Service

UV ..... Ultra Violet

VEU ..... Vaccine Evaluation Unit

VR ..... Variable Region

WCL ..... Whole cell lysate

Chapter 1 Introduction

1.1 Meningococcal Disease

Neisseria meningitidis is an important human pathogen and is a major cause of septicaemia and meningitis worldwide. Symptoms of meningitis, characterised as inflammation of the meninges, the membrane surrounding the brain and the spinal cord, include a rash, severe throbbing headache, stiff neck, photophobia, and fever, leading to confusion and coma[87]. Symptoms of septicaemia, characterised as an uncontrollable multiplication of bacteria in the blood, include rash, fever, chills, severe muscle pain in the back or thighs, joint pains and breathlessness[130].

Meningococcal disease can appear as either meningitis, septicaemia or a combination of both. This disease is known to develop rapidly and death in previously healthy individuals can occur within 24 – 48 hours from the onset of disease. High case fatality rates are associated with meningococcal disease (5 – 15 %) even with rapid diagnosis, and the prompt administration of antibiotics[117,159,293]. Mortality can be reduced with optimal intensive care and disease treatment[29], however up to 20 % of disease survivors are often left with severe debilitating tissue and neurological sequelae including mental retardation, loss of limbs and neurosensory hearing loss[81,219]. Age specific attack rates are highest in infancy and decline during childhood with a second peak in teenagers and young adults, conversely the rate of meningococcal carriage is lowest in infants and young children and highest in teenagers and young adults[109,256]. The three main causative pathogens of bacterial meningitis were *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae* and *N. meningitidis*[270]. However, the introduction of the Hib conjugate vaccine and the *S. pneumoniae* conjugate vaccine has resulted in declines in meningitis caused by these bacteria and *N. meningitidis* is now the leading cause of bacterial meningitis in the world[223,58].

Onset of meningococcal disease may occur 1 – 14 days following acquisition of the meningococcus, and it is only when the bacterium crosses through the mucosal epithelium into the blood stream that illness occurs[17,356]. Invasive infection with *N. meningitidis* can result in

various forms of disease including meningitis, severe sepsis and septic arthritis, pneumonia, purulent pericarditis and conjunctivitis, although the latter four presentations are rare[356]. Survivors of a meningococcal infection usually remain protected from further infection for life[163].

1.2 Meningococcal Carriage

The only known reservoir of *N. meningitidis* is the human nasopharynx and colonisation by meningococci, resulting in asymptomatic carriage as opposed to disease, is essential for meningococcal survival and the first step of pathogenesis[46,356]. *N. meningitidis* may be carried asymptomatically by as many as 5 – 25 % of the population at any time[49] with the rate of nasopharyngeal carriage lowest in young children (2 %) and highest (up to 25 %) in adolescents and young adults[109,43,286,302]. Carriage rates in the older population are less than 10 %[43,46]. Increased rates of meningococcal carriage have been found in closed populations such as military recruits and college and university students as a consequence of closed, crowded living conditions and mixing with new communities[43,287,36]. Other risk factors for meningococcal carriage include co-infections such as influenza and other respiratory viral infections, smoking and damage to the upper respiratory tract[288,36,183]. Social factors including attendance at pubs/clubs, and intimate kissing were all found to be highly associated with the risk of meningococcal carriage in British teenagers[183]. Carriage may be prolonged (lasting a few months), intermittent (lasting days to several weeks) or transient[35,286].

Carriage is an important part of the transmission of pathogenic strains of meningococci, where infection is usually from within the healthy population rather than from individuals with the disease[113,46,36]. Thus, an understanding of transmission of meningococci isolates is important for understanding the best way to manage the disease. Transmission of meningococci is via direct contact or inhalation of meningococci through very close and prolonged contact with droplets shed from the nose and the throat[87].

Carriage is itself an immunising event[114] leading to the development of bactericidal antibodies against capsular and noncapsular antigens[113]. Several studies have reported a correlation

between the detection of carriage and an increase in the detection of bactericidal antibodies against the colonising strain[152,153]. High levels of protective antibody may be produced for several months following clearance of the carriage strain[114,152]. However, whilst colonisation is beneficial in the induction of an immune response, carriage is not protective against colonisation with heterologous strains and is not always protective against invasive disease [8].

1.3 Microbiology

N. meningitidis is a Gram negative aerobic diplococcus[137], classified as a proteobacteria belonging to the kingdom Bacteria. It is further classified in the Neisseriales and Neisseriacaeae order and family respectively. Gram negative bacteria have a thin peptidoglycan cell wall situated in the periplasmic space between the inner and outer cell membrane (Figure 1.1). Gram positive bacteria have a thicker peptidoglycan cell wall but do not have an outer cell membrane. The outermost structure of meningococci is a polysaccharide capsule and is the target for mucosal and humoral immunity[113,114]. The polysaccharide capsule is used both as an epidemiological marker and a vaccine target[292].

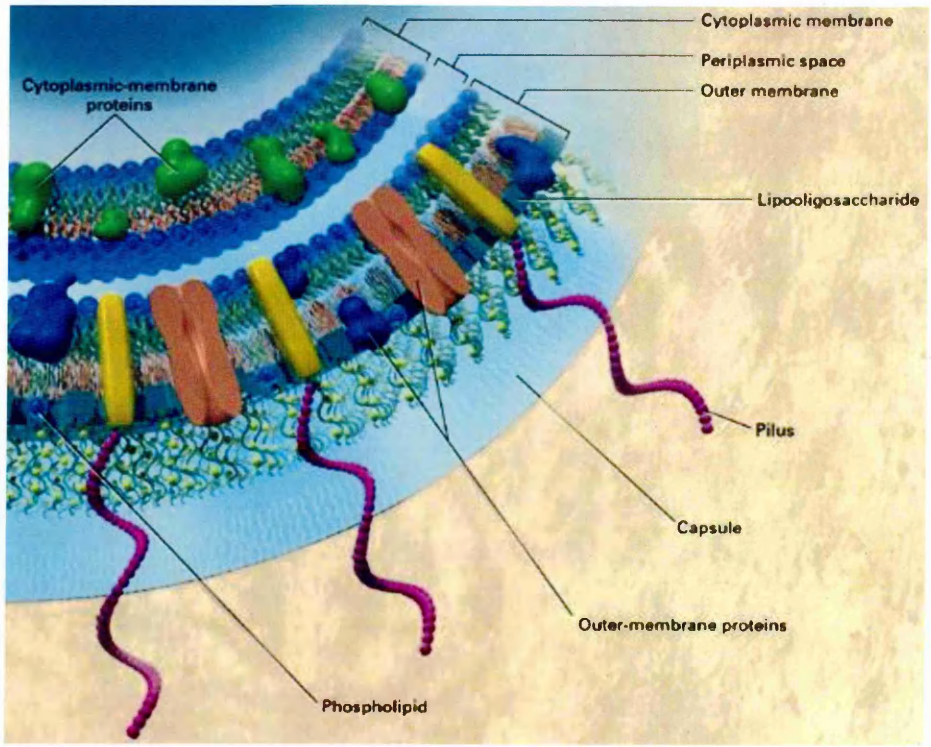


Figure 1.1 Surface structure of *N. meningitidis.* Source: Chiron Vaccines (http://meningitis.avenue-e.de/?idcat1=50).

The polysaccharide capsule is an important virulence factor of the meningococcus. Disease associated capsules have antiphagocytic properties essential for bacterial survival in the blood stream[(145)] affording the bacterium protection against environmental factors, therefore allowing growth and spread[(310)]. Conversely, expression of the capsular polysaccharide inhibits colonisation of the nasopharynx by masking meningococcal adhesins/invasins, suggesting that capsule expression may be down regulated during the initial stage of infection[(131)]. While the loss of capsule enhances the capability of meningococci to colonise the human nasopharynx and to avoid human defence systems, reports of disease caused by unencapsulated meningococci are very rare[(132,337)].

1.3.1 Capsule structure

The biochemical composition of the polysaccharide capsule is used to classify the species into twelve serogroups [(328)] of which five have been extensively studied. The serogroup A capsule is composed of repeating units of (α1→6)-linked N–acetyl-D-mannosamine-1-phosphate [(291)]. In contrast, serogroup B, C, Y and W capsules are composed of, or contain polysialic acid [(291)] and are

biochemically similar. Serogroup B and C capsules are polysialic acid homopolymers, but whilst the C serogroup contains both the N-and the O-acetyl groups, the B serogroup contains only the N-acetyl groups[177]. Serogroup Y and W capsules consist of sialic acid linked to D-glucose and D-galactose, respectively[308]. Serogroup B capsular polysaccharide is poorly immunogenic in humans and is structurally similar to the certain human glycoproteins. As a result, the perceived risk of autoimmune response is greatly increased if this capsular polysaccharide were to be used as a vaccine[221,231]. Notably however, no autoimmune disorders associated with antibodies to the serogroup B capsule polysaccharide have been reported[284,363]. Whilst increased titres of antibodies reactive to the serogroup B capsule have been observed following colonisation with a serogroup B meningococci, these responses have been transitory, and the subsequent decline in anticapsular antibodies was not accompanied by a corresponding decline in bactericidal activity[153]. This corresponds to previous studies where PorA is the major antigen associated with the production of a bactericidal immune response[347].

1.4 Epidemiology

Meningococcal disease is a significant worldwide health problem occurring as either a sporadic disease, affecting mainly children and adolescents, or in pandemic waves[223]. There are an estimated 500,000 cases per year, resulting in at least 50,000 deaths and a similar number of cases of neurological damage[22]. Epidemiology of meningitis is variable with morbidity ranging from 0.2 to 4.7 cases per 100,000 inhabitants in industrialised nations. In sub-Saharan Africa and the countries of the meningitis belt, epidemics involve from 100 up to 1,000 cases per 100,000 population[134].

Although meningococci can be classified into twelve different serogroups, only six serogroups are associated with 90 % of severe meningitis and septicaemia, i.e. serogroups A, B, C, Y, W and X[226,257,28]. Five of these serogroups have been studied extensively; however the emergence of serogroup X as a disease causing strain, and its ability to cause epidemics is a more recent discovery[28,72].

The different serogroups, whilst having a similar mode of action, have been shown to have differing effects, and have variable prevalence in different parts of the world[244]. Serogroup A is thought to be responsible for 80 - 85 % of meningococcal disease cases found in the sub-Saharan meningitis belt and Asia. Outbreaks of serogroup A disease tend to occur in irregular 8 – 10 year cycles, and last 2 – 3 years, predominantly peaking in March – April, at the end of the dry season, and dying out with the outset of the rainy season[133,22]. More than half the cases occur in individuals younger than 15 years of age. China, India, Nepal and Mongolia have also seen epidemics due to serogroup A meningococci[142,52,338]. Since World War II, large outbreaks of meningococcal disease caused by serogroup A have not occurred in Europe or the United States [245] and this strain is now rarely seen to cause disease in Western countries[127,134]. However, serogroup A meningococci have been associated with several epidemics within Europe since the 1960's. An epidemic in Russia from 1969 onwards was found to result from a spread of an epidemic strain of serogroup A meningococci from China and was associated with migration[6]. This strain persisted in Russia into the late 1990s. A serogroup A epidemic associated with an identical clone to that of the Russian epidemic was also experienced in Finland in 1973 – 1977[224], and in western Norway in 1969[138]. Serogroup A epidemics have also been reported in Greece in 1968[327] and Romania in 1970, with a second epidemic in Romania from 1985 – 1987[202]. An epidemic of serogroup A disease was reported amongst the indigenous population of central Australia from 1987 – 1991, and was found to closely resemble outbreaks in sub-Saharan Africa[220]. Serogroup B and C outbreaks are more common in Europe, the Americas and Australasia although incidence of serogroup C disease has decreased due to the use of the meningococcal serogroup C conjugate vaccine (MCC). Serogroup B accounts for 30 – 40 % of cases in North America, and up to 80 % of cases in Europe[273,244]. Serogroup C is responsible for the majority of the remaining cases. The case fatality and incidence of serogroup B and C disease are higher for young children and adolescents, than in newborns and the elderly in all countries[22]. The highest specific incidence of meningococcal disease occurred in infants <1 year of age[256], with a higher proportion of serogroup B disease observed in this age group in

comparison to cases due to other serogroups (Figure 1.2). However, a shift in the age distribution of meningococcal disease towards higher age groups (≥ 20 years of age) can be observed during outbreaks and epidemics[225,71]. Serogroup B isolates are responsible for severe persistent epidemics, many of which occurred in Latin American countries (Cuba, Columbia, Chile and Brazil); Norway and New Zealand. In many cases these have lasted for more than 10 years[45,262,16,22]. These periods of hyperendemicity are a helpful way of distinguishing between serogroup B infection activity and infections due to other serogroups, e.g. serogroup A. Disease caused by serogroup B meningococci is rarely seen in Africa and other developing countries.

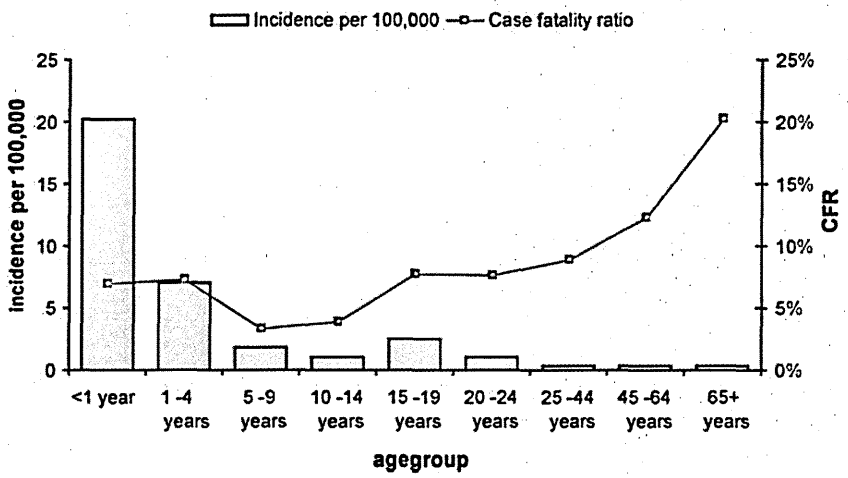


Figure 1.2 Incidence of meningococcal disease in Europe in 2006. Data is representative of 27 countries participating in EU-IBIS [134].

Serogroup Y meningococci have recently emerged as a causative agent, initially mainly in North America, since the 1990s[256]. Throughout 2006, enhanced surveillance has also revealed an increase in disease incidence due to serogroup Y in England and Wales[165], and northern Europe[136]. Whilst serogroup W organisms are a relatively rare cause of endemic disease, these have been associated with outbreaks, for example with the Hajj in 2000[7], and an epidemic in Burkina Faso in 2002[69]. Serogroup X meningococci were identified as the disease causing strain following a large epidemic of meningococcal disease in Niger in 2006[28].

1.5 Immunological Typing

Immunological approaches were the first methods to be widely accepted for meningococci typing[101]. These included the identification of the serogroup, based on the meningococcal capsule[328], serotypes and serosubtypes, based on the outer membrane proteins (OMPs) PorB and PorA, respectively[98]. For example a strain with serogroup B, serotype 2a and serosubtype P1.5,2 would be designated B:2a:P1.5,2. The immunotype, based on the lipopolysaccharide (LPS) can also be used for further strain resolution[306] however this is now not commonly used (Figure 1.3).

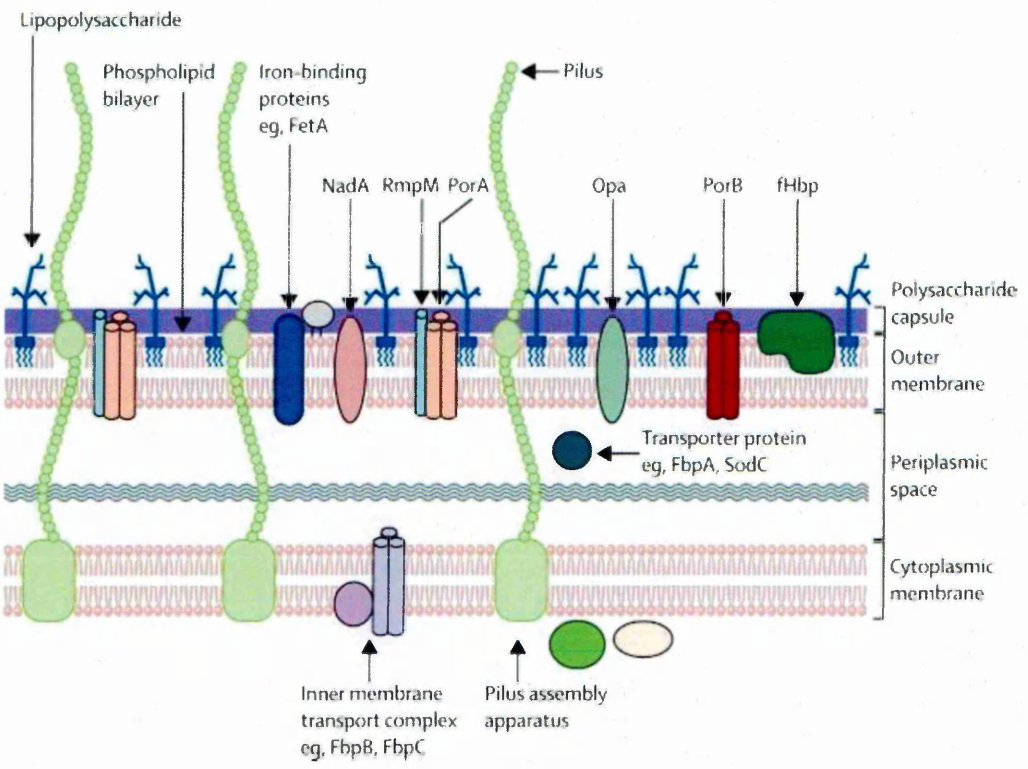


Figure 1.3 Surface structures of *N. meningitidis* including those used for serological classification[263].

1.5.1 Outer membrane proteins

The outer membrane of the meningococcus consists of a lipid bilayer, embedded with a complex range of membrane proteins essential for selective permeability and for maintaining the structural integrity of the membrane. In early studies meningococcal OMPs were categorised in five main classes, designated class 1 to class 5, based on differences in molecular weight[305,99].

The class 1 and class 2/3 proteins are the most abundant and immunogenic OMPs in pathogenic *Neisseria* species, and are found in all Gram negative bacteria[143]. PorA, previously known as the class 1 protein, and PorB, previously known as the class 2/3 protein act as selective channels, allowing the passive passage of ions across the cell membrane[101,143]. Although the function of the class 4 Reduction modifiable protein (Rmp) is not known, it is suggested that RmpM is involved with peptidoglycan binding, acting as a bridge to anchor the peptidoglycan to the outer membrane through the formation of stabilized heterooligomeric complexes with PorA and PorB[143]. RmpM is also associated with iron regulated proteins, lactoferrin binding protein (LbpA), transferring binding protein (TbpA) and FetA (also known as FrpB), again resulting in the formation of oligomeric complexes[243]. Antibodies directed against this protein are not bactericidal[210]. The heat modifiable class 5 opacity proteins, Opa and Opc play an important role in the adhesion and entry of both *N. meningitidis* and *N. gonorrhoeae* to endothelial and epithelial host cells[334,333,68]. Opc proteins have been shown to bind different members of the carcinoembryonic antigen receptor family, present on endothelial, certain epithelial and polymorphonuclear cells[335,336]. Opa proteins in comparison have been shown to interact with the serum glycoprotein vitronectin, using this protein to attach to heparin sulphate proteoglycans present on the endothelial cells[68]. Expression level of Opc varies between meningococcal strains, with high-level or intermediate-level expression observed in some strains and no Opc expression observed in others[268]. Whilst the *opc* gene is found in gonococci, Opc is only expressed in meningococci[361]. In contrast, Opa is expressed in most meningococcal and gonococcal isolates, with up to four distinct Opa proteins expressed by the meningococci and up to 11 distinct Opa proteins expressed by gonococcal isolates resulting in a heterologous population of bacteria expressing none, one or multiple Opa proteins[164]. The outer membrane also contains LPS which helps to confer serum resistance and is involved in the pathogenesis of meningococcal disease[208].

1.5.1.1 PorA

The immunodominant antigen of *N. meningitidis* is the highly variable OMP, PorA[323,64]. PorA is a 44 – 47 kDa cation-selective transmembrane protein which forms a trimeric pore in the outer

membrane of meningococci. A transmembrane 16 strand β-barrel conformation has been proposed for PorA based on known *Escherichia* porin structures[318] with eight surface exposed loops (I – VIII). Two distinct variable regions (VR), VR1 and VR2 corresponding to loops I and IV, respectively, are located on the longest of these loops (Figure 1.4)[198,188,318] and form the basis for serosubtyping of *N. meningitidis.*

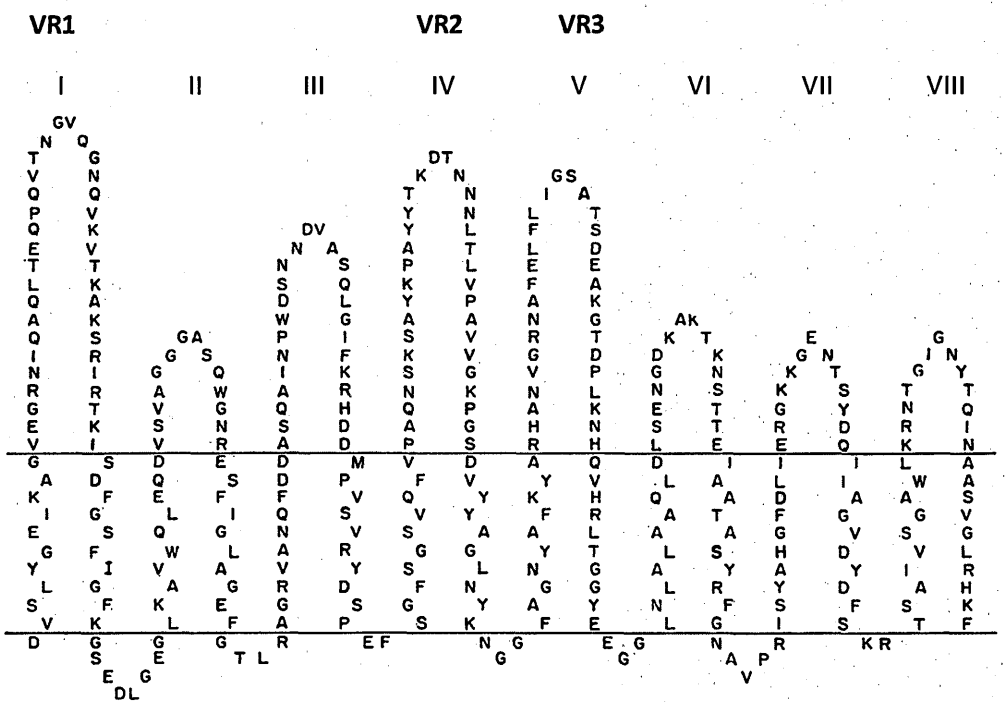


Figure 1.4 Proposed structure of the PorA protein with a P1.21, 16 serosubtype. The top part of the model show the surface exposed regions, whilst the central part indicates the transmembrane regions[318].

The PorA protein, is assigned the "P1." prefix and is expressed as a pair of numbers corresponding to the subtype on the two variable regions (VR1 and VR2) located on the surface exposed loops of the PorA protein[261,258]. The reaction of monoclonal antibodies (MAbs) with specific regions of VR1 and VR2 have previously been used to serosubtype *N. meningitidis*[2], however meningococci were frequently only partially serosubtyped or classified as non-serosubtypeable. This was due mainly to a lack of a comprehensive panel of PorA MAbs[258]. Nowadays molecular methods are used to subtype meningococcal isolates (http://pubmlst.org/*Neisseria*/). The amino acid sequences of VR1 and VR2 deduced from nucleotide sequence data are used rather than relying

on reactivity with specific MAbs[258] Amino acid sequences with ≥80 % similarity to each other were grouped into VR families, with the VR epitope recognised by an existing MAb raised against PorA, or the first defined amino acid sequence of a VR family designated as the prototype VR for that family[258].

A third region of variability (VR3), located on loop V, has a lower level of genetic variation[61] but this region is not thought to be as immunologically important as VR1 and VR2. Loop V is a shorter loop than either loops I and IV and therefore maybe less exposed to the immune system and thus selection pressure, however bactericidal antibodies to this region have been produced[61,62]. A number of VR3 genosubtypes have been described[51] therefore VR3 may be sequenced along with VR1 and VR2 to allow for further characterisation of PorA.

1.5.1.2 PorB

The second major porin is PorB and is expressed by all meningococci[101] and makes up the vast majority of water-filled channels within the Neisserial outer membrane[356]. Pores formed by the PorB protein are thought to be anion-selective[143]. Topology models have been constructed for PorB based on nucleotide sequence data[188,318] and three dimensional homology models have been generated using structural similarity between *Escherichia coli* porins and Neisserial porins[74]. Like PorA, the 37 – 42 KDa PorB protein is predicted to consist of a transmembrane β-barrel conformation with eight surface exposed loops. Whilst some serotypes can be assigned to particular peptide sequences[311,312], the PorB serotype epitope is largely discontinuous, and made up of several of the eight surface exposed variable regions which, when the protein is in its native conformation, are in close proximity[357].

Serotyping was previously based on the reactivity of MAbs against immunogenic regions within the PorB protein; however, as for PorA, sequence based typing is more commonly used to reduce the number of non-typeable meningococci[2,3,261,80]. PorB genotyping is most commonly used where further resolution is required, however this is not used in the recommended nomenclature of *N. meningitidis*[149,180].

1.5.1.3 FetA

Iron acquisition is a well defined determinant of bacterial pathogenesis, with the production of several ligand-specific iron transport proteins involved in the acquisition of iron from the host binding compounds. *Neisseria* acquire iron through direct interaction between the iron binding glycoproteins such as lactoferrin, transferrin, haem, haemoglobin, and haemoglobin complexed to the serum glycoprotein haptoglobin and the bacterial cell[37,355,209,173] via receptors specific for human lactoferrin and transferrin on the meningococcal surface[307,83]. One such iron binding protein is FetA (also known as Frbp), a member of the TonB dependant class of OMPs [60].

Meningococcal FetA is a 70 kDa iron–regulated OMP with a predicted β-barrel configuration located in the outer membrane with 26 membrane spanning domains and 13 surface exposed loops[162]. As with PorA, the longest loop (loop VII) contains the immunodominant region corresponding to a single hypervariable region of the protein[296]. FetA was not routinely used as a serological typing target, as it is only expressed under conditions of iron limitation, and so whilst it will be expressed in the human host, *in vitro* expression is dependent on iron limitation[149]. However, FetA is a major component of the outer membrane and the *fetA* gene is found in most meningococcal isolates. Following on from the introduction of sequence based typing schemes, antigenic sequence typing of FetA is part of the molecular typing scheme for *N. meningitidis*[149].

1.5.2 Typing at a population level

The clonal nature of relationships among *N. meningitidis* isolates has been demonstrated, and whilst some clones have been repeatedly isolated worldwide, others have rarely been isolated[241]. Meningococci associated with epidemics and outbreaks generally belong to distinct clonal groups, whereas sporadic meningococcal disease is caused by more diverse meningococci[4]. A number of techniques have been used to investigate genotypic relationships among meningococci. These include pulsed-field gel electrophoresis (PFGE)[8,241], amplified fragment length polymorphism (AFLP)[122], multilocus enzyme electrophoresis (MLEE)[47], and multilocus sequence typing (MLST)[185].

MLEE can differentiate between isolates by identifying the variation in the electrophoretic mobility of nine cytoplasmic enzymes using starch gel electrophoresis[44]. Electrophoretic types (ETs) are assigned based on the multilocus profile of the isolate, with only isolates that are marginally different assigned to the same clonal group or complex. Several hypervirulent, hyperendemic ETs have been identified using MLEE and include ET37, ET5 and the A4 complex[338,122].

MLST was developed as an alternative to MLEE, with nucleotide sequences used to characterise isolates of bacterial species using seven housekeeping gene fragments[185]. Housekeeping genes are used in MLST as they encode proteins required for bacterial growth, and so are constantly expressed, and are under non-diversifying selection[244]. Alleles are assigned to each housekeeping gene, within a bacterial species, based on different nucleotide sequences, and the seven loci are used to assign a sequence type (ST) to each isolate. This provides unambiguous sequence data allowing for high levels of discrimination between isolates and for the rapid identification of hypervirulent meningococci without the need for culturing of the organism[84]. The number of nucleotide polymorphisms is not important in MLST, instead sequences are given a different allele number whether they vary at a single or multiple nucleotide sites. The MLST database is then used to analyse sequence data unambiguously against a large central database (http://pubmlst.org/*Neisseria*/)[150]. The majority of disease causing isolates fall into distinct clonal complexes and are monitored across Europe by the European Centre for Disease Prevention and Control.

The analysis of six to eight housekeeping gene fragments, as utilised for MLST is not always suitable for the resolution of closely related organisms[5]. Extended MLST (eMLST), which uses an increased number of housekeeping genes, in addition to dispensable genes sequences can help to further resolve genotypic differences within clonal complexes. However, many variations between the genotype and phenotype occur at a level other than at the level of clonal complex, and cannot be resolved using eMLST[75]. In addition, due to the diversity of bacterial organisms, a

separate MLST scheme is required for each group of related bacteria, and MLST typing schemes are limited to bacteria belonging to the same genus[184].

With the introduction of rapid nucleotide sequencing schemes, the number of whole bacterial genomes available for analysis has greatly increased[201], allowing for analysis of genetic variation across the whole domain at selected shared loci[148]. Using the Bacterial Isolate Genome Sequence Database ($BIGS_{DB}$)[151] a combined taxonomic and typing approach has been introduced for the whole domain bacteria. Ribosomal MLST (rMLST) indexes the sequence of ribosomal protein-encoding genes, into a MLST scheme providing increased resolution of the whole domain bacteria to the species or subspecies level[148].

1.6 Vaccines

A major task for vaccine manufacturers is to design a meningococcal vaccine that is safe, addresses individual or public health issues, and is effective in the age groups most susceptible to disease. There are many different types of meningococcal vaccine available, but none currently offer protection against all serogroups in all age groups. A problem in the development of vaccines is due to the peak incidence of meningococcal disease being in children under 1 year of age[97] and the poor immunogenicity of carbohydrate antigens such as the capsular polysaccharides in the very young[171]. Whilst polysaccharide vaccines offer protection in older age groups, protection is serogroup specific[107]. Conversely, whilst protein vaccines are more immunogenic than carbohydrate vaccines, the immunogenic meningococcal surface protein antigens are also highly antigenically variable, thereby limiting the cross protection afforded by the vaccine.

1.6.1 Polysaccharide vaccines

The first meningococcal vaccines, based on purified capsular polysaccharide were developed in the 1960s and shown to protect against meningococcal infection in humans[119]. Plain polysaccharide vaccines are safe and systemic reactions are rarely seen[111,110]. Both the serogroup A and C polysaccharides are good immunogens in humans and the first successfully

developed capsular vaccines targeted these polysaccharides[119]. The efficacy of the group C capsular vaccine was initially shown in military recruits and later used to prevent an outbreak of group C meningococcal disease in the US Army[97]. Immune responses to both vaccines were age-dependant with short term efficacy levels of 85 – 100 % observed in older children and in adults. However, neither vaccine was found to elicit protective antibodies in younger children and infants, with no response detected to the serogroup A vaccine, and a low level response to the group C vaccine in infants immunised at three months of age[110]. This is thought to be due to the poor immunogenicity of the capsular polysaccharides in children under two years of age.

1.6.2 Conjugate vaccines

Conjugate vaccines consist of the saccharide antigen covalently bound to a T-cell dependant carrier protein, such as tetanus toxoid or CRM197[112]. There are several benefits of conjugate vaccines over polysaccharide vaccines. These include: the induction of herd immunity; longer lasting protection through the induction of immunological memory; the induction of high avidity antibodies; effectiveness in infants and young children; and the ability to boost the immune response with repeated doses of vaccine with less risk of a hyporesponsive immune response [124,26]. Conjugate vaccines have been found to be effective in reducing the acquisition of carriage and disease across all age groups, including children less than two years of age, with vaccine efficacy demonstrated in infants as young as two months of age[124]. The first conjugate vaccine developed was the Hib polysaccharide-tetanus toxoid conjugate vaccine and has been implemented into routine infant immunisation schedules in over 30 countries[352].

Following the success of the Hib vaccine, a number of meningococcal group C conjugate (MCC) vaccines have been developed and have considerable advantages over polysaccharide vaccines. There are three MCC vaccines licensed globally[250]. All three vaccines, were found to induce bactericidal antibodies and immunological memory in UK toddlers after one dose[250], and in infants following three doses[251,18]. The MCC vaccine was introduced into the UK infant vaccination schedule in 1999, with a simultaneous two-dose catch-up programme offered to

infants aged between five and eleven months, and a single-dose catch up offered to children aged 1 – 17 years of age in order to induce widespread immunity[205]. Introduction of the MCC vaccine led to a dramatic reduction in disease cases caused by serogroup C strains of *N. meningitidis* (Figure 1.5)[18,39]. By 2006, the MCC had been introduced into routine immunisation schedules across Europe, with a resulting 10-fold reduction in the incidence of meningococcal serogroup C disease[105].

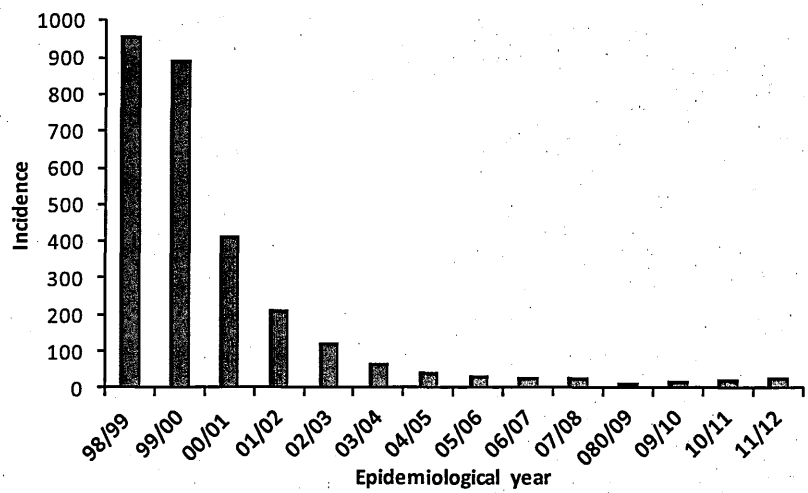


Figure 1.5 Incidence of invasive Serogroup C meningococcal disease in England and Wales following introduction of the MCC vaccine in 1999 (adapted from epidemiological data reported by the Health Protection Agency (HPA))[1].

As with the Hib vaccine, the effectiveness of the MCC vaccine began to wane over longer periods and the age at which the final dose of the vaccine was given, was found to be important in vaccine effectiveness. The effectiveness of the vaccine remained high in children targeted in the catch-up campaign (five months to 18 years), however, direct protection was not found to persist for longer than one year in children immunised at a younger age[299]. As a result, the MCC vaccination schedule was changed in 2006, to two doses at three and four or five months, with a booster dose at 12 months of age[38]. In order to afford greater protection to teenagers and to make the make the overall MCC vaccination programme more effective, the vaccination schedule was again changed in 2013. A single priming dose at three months of age was found to be sufficient to

1 http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/MeningococcalDisease/EpidemiologicalData/

provide protection against meningococcal serogroup C disease in the first year of life[89]. A booster dose given at 12 months of age, and a second booster dose at 14-15 years of age will provide higher levels of antibody that persist for longer[299,31] and persisting herd protection will maintain disease control for a number of years[301,31,105]. Despite the concern of capsule switching, where the type of capsule expressed by meningococcal clones changes, no evidence has been found with the use of this vaccine[39].

Several conjugate vaccines have also been developed for serogroups A, W and Y in a range of formulations. Quadrivalent vaccines, targeting serogroups A, C, Y and W, have been developed and are currently in use in the US, Canada and Europe[227,248,218]. A bivalent vaccine (Menhibrix), targeting both serogroup C and Y meningococci in addition to Hib has been licensed in the US for use in infants as young as two months of age[212] (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6203a3.htm). In 2010 Burkina Faso was the first country to introduce a new serogroup A conjugate vaccine (MenAfriVac) to eliminate epidemic meningitis in Sub-Saharan Africa[76].

With the availability of vaccines against serogroups A, C, Y and W, the greatest challenge is now the development of a serogroup B vaccine, however there is an increased risk of the induction of autoimmune antibodies due to the poor immunogenicity of the serogroup B capsule and the antigenic similarities with human neural cell adhesion molecule[233,91,231]. For this reason, vaccines based on capsular polysaccharides do not offer any protection against serogroup B meningococci.

1.6.3 Outer Membrane protein and vesicle vaccines

As the feasibility of a serogroup B capsular polysaccharide vaccine has been questioned[125], the use of other vaccine targets has been considered, and the focus for the development of vaccines offering protection against serogroup B organisms has for many years been non-capsular antigens[97,237,231,140]. A number of OMPs, shown to induce bactericidal activity against different meningococcal isolates have been investigated[269,97,295] and the PorA protein was the most immunogenic[253]. The use of LPS-depleted OMPs was considered a good approach for the

development of an effective serogroup B meningococcal vaccine and led to the development of several vaccines from single strains of serogroup B meningococci[147]. However, the efficacy and/or the effectiveness of these vaccines have not been completely satisfactory[147,115] with vaccines limited by poor immunogenicity in infants[204], and the short duration of immune response[27]. The poor performance of these vaccines was largely attributed to the loss of tertiary structure that accompanied precipitation and recent research into serogroup B vaccines has concentrated on the development of outer membrane vesicle (OMV) vaccines with proteins displayed in their native confirmation[208].

1.6.3.1 Outer membrane vesicle vaccine

Outer membrane blebs, released naturally by many Gram negative bacteria during bacterial growth, are small particles of a Gram negative cell wall. These blebs consist of OMPs, LPS and phospholipids (PL) as well as periplasmic proteins as they would normally be situated in a bacterium, on a much smaller scale[314];[24,314]. Outer membrane blebs have a range of functions in bacterial survival including mediation of bacterial envelope stress, biofilm formation, virulence and transformation[55]. Outer membrane vesicles (OMVs) produced as a result of detergent extraction of OM blebs, have reduced levels of LPS and are ideal candidates for vaccine delivery due to their immunogenic properties, ability to be taken up by mammalian cells and potential for recombinant engineering[55].

OMV vaccines based on single strains of serogroup B meningococci have been developed for use in areas with clonal meningococcal outbreaks, including Cuba, Norway and New Zealand[255,86,217]. These vaccines were shown both to elicit serum bactericidal antibody (SBA) responses and to protect against the development of meningococcal disease in clinical trials[65,27,276,231]. Overall efficacy of these vaccines was between 50 – 80 %, however, in some studies no protection was observed in young children[65,213]. The PorA protein is considered an important candidate of meningococcal vaccines[317] as the immunodominant antigen PorA proteins are a major inducer of SBAs[295]. However, PorA is highly variable among isolates, with the low cross reactivity of

antibodies between serosubtypes limiting the use of PorA as a cross protective antigen[253,324,161]. In order to produce a vaccine providing widespread coverage across a diverse range of organisms, a number of PorA proteins representing the most common variants must be included[297,330]. In spite of the variable nature of FetA, this protein also has potential as a vaccine candidate due to its ability to induce high levels of bactericidal antibodies[347,161]. OMV vaccines include FetA along with other antigens such as PorA and PorB as vaccine components[100]. These vaccines have been shown capable of inducing bactericidal responses in people, including infants, however this response is strain-specific[192] and can probably be attributed to PorA as this is the immunodominant antigen in OMVs. Limitations of OMV vaccines include waning immunity, protection falling after eight months to one year following a two-dose immunisation schedule with no booster, and strain/serosubtype restricted immunity in infants[295]. OMV vaccines based on single meningococcal strains are beneficial in the fight against clonal disease outbreaks, but are not beneficial for sporadic endemic disease caused by differing meningococcal strains.

Two of the most extensively studied vaccines were produced in the 1980's in response to meningococcal outbreaks in Cuba (VA-MENGOC-BC®) [276] and Norway (MenBvac™)[27]. The Cuban OMV vaccine, produced at the Finlay Institute from a B:4:P1.15 strain of *N. meningitidis*, is a bivalent vaccine composed of serogroup C capsular polysaccharide and serogroup B OMVs with a P1.19,15 serosubtype[276,208,309]. VA-MENGOC-BC® was licensed for use in 1989 and has been used successfully to control epidemics in Cuba, Brazil, Columbia and Uruguay[65,34,280]. The efficacy of this vaccine was found to be age dependent, with estimated efficacies of 37 % (95 % CI: 100-73) in infants under 24 months of age, 47 % (95 % CI: 72-84) in children aged 24 – 47 months and 74 % (95 % CI: 16-92) in children aged over 48 months[204]. A high level of efficacy (83 %) was demonstrated in a large clinical trial following a two-dose schedule of the vaccine in school children aged between 10 – 14 years of age. The vaccine was not found to have any efficacy in children less than 24 months of age[204]. The presence of antibodies to cross-reactive epitopes was detected following immunisation with the VA-MENGOC-BC vaccine[203].

The high incidence of serogroup B meningococci disease observed in Norway during the 1970s and early 1980s motivated the development of an OMV vaccine against a strain representative of the epidemic (B:15:P1.7,16)[102,253,208]. The MenBvac vaccine was produced at Norwegian Institute for Public Health (NIPH), is safe, immunogenic and confers protection against group B meningococcal disease[27,253]. The efficacy of this vaccine, as for the Cuban vaccine, was found to wane and studies of a two-dose regime in teenagers over 29 months from 1988 – 1991 estimated the efficacy at 57 %[208], although a three-dose regime gave a longer, more cross-protective response[295,267]. Vaccine efficacy among children (from 12 to 16 years of age) was found to be too low for general use of the vaccine[27]. A combination of the MenBvac vaccine with a MCC vaccine was demonstrated to be immunogenic with regard to both serogroups B and C meningococci in adult volunteers[1].

An epidemic of systemic serogroup B meningococcal disease in New Zealand caused by a single serosubtype (P1.7-2,4)[191], accounted for an estimated 80 % of all meningococcal disease cases at its peak[274,172]. A similar approach to vaccine development was adopted as for MenBvac, and a tailor-made vaccine was developed by the Norwegian Institute of Public Health (NIPH) in Norway and Novartis, in partnership with the New Zealand Ministry of Health and the University of Auckland[216]. MeNZB is a meningococcal serogroup B OMV vaccine administered in a three-dose regime. With a seroresponse, defined as a four-fold rise in serum bactericidal antibodies, found in 96 % of adults, 76 % of children, 75 % of toddlers and 74 % of infants receiving three doses of MenZB, the vaccine was safe and efficacious in adults and children of all ages and licensed for use in 2004[216]. In order to achieve rapid epidemic control, a large randomized controlled efficacy test was not undertaken for the vaccine, following satisfactory safety and immunogenicity profiles demonstrated in phase I and phase II trials and following experience of other group B OMV vaccines extensively used elsewhere, however, a prospective observational study demonstrated a vaccine efficacy of 73 % (95 % CI 52 – 85)[156]. The vaccine was found to be particularly effective in the younger population with efficacies of 83 % (95 % CI: 59.4 – 94.3) in children between 0.5 – 3 years of age and 80 % (95 % CI: 52.5 – 91.6) in children between 0.5 – 5 years of age[104].

As with other OMV vaccines, the protective response was not long-lasting and poor immunogenicity was observed in young infants aged between six to eight months with four doses required to produce a protective response[217,172]. The use of an intensive immunisation campaign, offered to all young New Zealanders under the age of 20 years of age (2004-2006), with three doses of vaccines affording 80 % coverage has led to the effective management and control of New Zealand's meningococcal epidemic[172]. OMV vaccines such as MenBvac and MeNZB have been found to reduce meningococcal carriage and may therefore induce herd immunity[70].

To overcome serosubtype restricted protection afforded by single strain OMV vaccines, the National Institute for Public Health and the Environment (RIVM), Netherlands, developed a multivalent vaccine comprised of PorA proteins from six different prevalent pathogenic isolates[319,323,50]. Hexamen is a recombinant, hexavalent vaccine composed of two OMVs produced using two genetically engineered variants of the H44/76 vaccine strain, each expressing three PorA serosubtypes. The first strain, PL16215 encodes for the P1.7,16, P1.5-1,2-2, P1.19,15-1 serosubtypes and the second strain, PL10124 for the P1.5-2,10, P1.12-1,13, P1.7-2,4[50,323]. The six PorA proteins in the vaccine were represented in over 80 % of UK and western Europe meningococcal case isolates in 1996 to 1999[323,50,42,208]. Hexamen was safe, well tolerated and induced bactericidal antibodies against strains expressing each of the six PorA proteins in adults[222] and following a three-dose immunisation schedule in infants[42], toddlers and children[64]. As for the previous OMV vaccines, bactericidal response in infants quickly waned, however a fourth dose at 12 months boosted serum antibody titres to protective levels[42].

To provide an even broader range of protection, Hexamen was expanded to include a third trivalent vaccine strain, HP1416, making up the nonavalent PorA OMV vaccine, Nonamen. The serosubtypes included in this vaccine are the P1.7,16; P1.5-1,2-2; P1.19,15-1; P1.5-2,10; P1.12-1,13; P1.7-2,4; P1.22,14; P1.7-1,1 and P1.18-1,3-6[316]. Together the nine PorA proteins in the vaccine represent the most frequently occurring serosubtypes in industrialised countries[316]. Nonamen has been showed to be immunogenic in mice and rabbits. A Phase I trial was carried

out in Spain in 2007 and as with HexaMen, NonaMen was found to be safe and well tolerated, and on the basis of European PorA subtype data from 1999 to 2004, is predicted to have a potential coverage of 80 % based on European PorA subtype data from 1999 to 2004[304].

Traditionally, OMV vaccines are prepared using detergent extraction to remove LPS, and increase vesicle release. However, whilst selective removal of LPS in this way reduces endotoxicity, the PL and lipoproteins, such as factor H binding protein (fHbp) that contribute to immunogenicity, are also removed[160]. Immune responses resulting from detergent extracted OMV vaccines are therefore directed against specific PorA subtypes and do not provide cross-protective responses[324,208]. A number of methods have therefore been investigated for the detergent-free purification of OMV. Vaccines produced using detergent-free OMVs have shown promising results in animals and humans with cross-protection against a panel of serogroup B strains but are limited by the high LPS content to intranasal administration[139] and the low yield of OMV following purification[88]. A new generation of OMV vaccines has been developed utilising meningococcal strains with genetically attenuated[283] or detoxified LPS[320]. This allows native OMVs (NOMV) to be used without detergent extraction needed to remove LPS[321], reduced OMV aggregation, and comparable yield and toxicity to detergent extracted OMVs[315]. Retention of membrane associated lipoproteins in the NOMV allows proteins such as fHbp and transferring binding protein, to be present in the vaccine[363]. An advantage of using a vaccine with multiple antigens is the potential for a synergistic bactericidal action of antibodies induced to the different antigens[353,351]. Vaccines produced using NOMV have been shown to produce a broad based bactericidal antibody response against serogroup B meningococcal strains in mice and humans[315,232]. The bactericidal antibody response induced by a trivalent NOMV vaccine in mice is also effective against serogroups C, Y, and W, epidemic serogroup X, and certain serogroup A strains of meningococci in mice[232]. An improved next generation Nonamen has also been produced and studies in rabbits and mice have found the vaccine to be safe and have the potential to enduce a protective immune response[154].

1.6.4 Reverse vaccinology

Reverse vaccinology is a method of using a bioinformatic approach to screen the entire genome, to identify and investigate meningococcal proteins as putative vaccine candidates[247] and was developed following publication of the genome sequence of the virulent MC58 strain of serogroup B meningococci. DNA fragments were analysed to reveal open reading frames (ORFs) and up to 600 novel genes predicted to encode surface exposed or exported proteins were identified. These genes were amplified and cloned into *E. coli* for expression as fusion proteins. Recombinant protein was successfully purified from 350 of these genes and used to immunise mice. The resulting sera were analysed to confirm the surface exposure of the proteins and the ability of the protein to induce bactericidal antibodies. Of these, 85 proteins were found to be novel surface-exposed proteins, of which 25 proteins induced bactericidal antibodies. Finally, seven proteins were selected for further analysis[233,247].

One such antigen identified through reverse vaccinology is the fHbp[195,95]. This is a surface exposed lipoprotein expressed by most meningococci. Factor H is an important regulator of the alternative complement pathway and fHbp recruits human factor H to the bacterial surface to help it to evade complement-mediated killing[272]. Expression of fHbp may vary between strains, but the genetic sequence for this protein appears to be present in almost all strains of *N. meningitidis*[195,95,20,289]. Other antigens discovered by reverse vaccinology include Neisserial adhesion A (NadA) and Neisserial heparin-binding antigen (NHBA)[108].

The first vaccine produced using this approach is the four component formulation (4CMenB), known as Bexsero®, and was licensed for use in Europe in January 2013 and more recently in Australia. This vaccine is made up of four components, fHbp (variant 1.1), NadA (variant 3.1), NHBA (variant 1.2) and a single serotype OMV (expressing PorA P1.4)[289]. Bexsero® was found to be safe, well tolerated and immunogenic in adolescents, adults[157], and in infants[90,279,331]. More than 95 % of infants aged six to 12 months generated a SBA response as early as one month post-second dose against strains representing multiple antigens included in the vaccine[279]. Although a

decline in the level of protective antibodies was observed in infants, six months following the third dose, a booster dose resulted the restoration of protective levels in 93 % of participants[289,331]. Bexsero has a predicted vaccine coverage of 78 % (95% CI 66-91) of meningococcal B strains in Europe[77] 76 % (95% CI, 59% to 87%) of strains in Australia[118] and 66 % (95% CI: 46-78%) of strains in Canada[23].

1.7 Vaccine Evaluation

Immunity against invasive meningococcal disease is dependent on bactericidal activity by serum immunoglobin G (IgG), together with complement[113,144]. The importance of IgG in protection against meningococcal disease is highlighted by the inverse relationship between antigen specific serum IgG levels and incidence of invasive disease[113]. Conversely, antigen specific serum IgG levels remain low until adolescence and then increase steadily, while carriage rates of meningococci rise rapidly in childhood before steadily falling with increasing age[163]. The bactericidal antibody response produced in response to carriage, provides high levels of protective antibodies, specific to the carriage strain and also cross-reactive to heterologous strains[249] through shared antigens including PorA and PorB. However unlike the immune response elicited by invasive disease, meningococcal carriage does not always elicit a protective immune response against meningococcal disease and any protection may be short lived[8]. Bactericidal activity against meningococci is dependent on the IgG class of antibodies[120].

Various serological methods have been used to measure IgG responses to capsular polysaccharides including capillary precipitation[121], bactericidal antibodies[113], opsonisation[252], immunofluorescence[113], passive hemagglutination[121], solid-phase radioimmunoassay[362], solution-phase radioimmuno assays[155] and enzyme-linked immunosorbent assay (ELISA). Of these, only the radioimmunoassay and ELISA were found to accurately determine total anti-polysaccharide antibody levels using the small volumes of sera typically obtained from young children[40]

1.7.1 Serum Bactericidal Antibody Assay

Correlates of protection are used for the evaluation of vaccines when a low sample population is available, due to low disease incidence, previous vaccination of subjects, or where vaccine challenge may be unethical. The correlate of protection used for evaluation of meningococcal vaccines is the SBA assay. This assay is considered to be the gold standard for determining meningococcal vaccine efficacy with standardised SBA assays developed for serogroups A, B and C[196,32]. The ability of SBAs to confirm protection against meningococcal disease is widely accepted, and, for purposes for licensure of meningococcal vaccines, regulatory agencies generally accept induction of these antibodies as evidence of vaccine effectiveness[32,207]. However, this is a complex method with a number of variables that have to be considered in the design of the assay, including the use of live bacteria and the source of both complement and serum. It has also been suggested that the extent of meningococcal immunity in the population, in addition to the potential effectiveness of new meningococcal vaccines are being underestimated due to the reliance on SBA assay results[350].

Extrapolation from measurement of total serum anticapsular antibodies to predict bactericidal antibody titres may be limited[123]. For example, serum from young children and infants vaccinated with a meningococcal polysaccharide vaccine were found to have high serum antibody responses to the serogroup C polysaccharide, however, no bactericidal antibodies were detected[176,197]. Although the immunogenicity of some meningococcal vaccines have been described in terms of the presence of polysaccharide binding antibody concentration, only bactericidal titres measured using SBA assay are associated with protection from meningococcal disease[113,204,197].

1.7.2 Enzyme-Linked Immunosorbent assay (ELISA)

A standard ELISA based protocol was first proposed for the measurement of total meningococcal serogroup A antibodies[40] and for serogroup C antibodies[106] following the development of polysaccharide-protein conjugate vaccines. Use of the ELISA as a standardised method for the

quantification of total meningococcal polysaccharide antibodies was selected as it was simple, sensitive, reproducible and specific for anticapsular antibodies[40,106]. ELISA methods for the detection of antibodies against strain specific epitopes and outer membrane proteins of N. meningitidis for serotyping and serosubtyping[2] have been developed and are being used for measurement of vaccine induced IgG responses. ELISA has been used for the quantification of total anti-OMV antibodies following vaccination with an OMV vaccine, with the vaccine strain OMV used as the coating antigen[116]. The ELISA is well suited to the screening of many samples for a single analyte[167]. The inability to multiplex a standard ELISA means a separate ELISA is required for the measurement IgG responses produced for each analyte of interest[167]. This limits the use of ELISA in the evaluation of vaccines containing multiple antigens of the meningococcal outer membrane. For example, determining the immune response resulting from vaccines such as HexaMen and Nonamen would require six and nine separate ELISAs, respectively. Therefore use of ELISA for quantification of immune responses to multiple analytes is a time consuming process; often costly, both in terms of serum volume and intensity of labour[67], particularly as the number of target antigens in vaccines are increasing[167].

ELISA is used for quantification of antibody responses to vaccination and disease, these results do not always correlate well with functional antibody assays[123]. Good correlation with the SBA assay is observed particularly when assaying sera from immunised adults[10] or in teenagers and young adults following the administration of a single vaccine[158]. The SBA assay and ELISA correlate less well in sera from infants or toddlers[171], or with sera from studies comparing polysaccharide vaccine to a conjugate vaccine[176]. Differences in the avidity of the antibodies measured are likely to be the cause of differences in correlation between SBA assays and ELISA[123]. A modified ELISA was developed to ensure specificity, and favour the detection of high avidity antibodies[123] and was found to correlate well with SBA assays.

Other examples of modified ELISA include indirect whole cell ELISA and dot-blot ELISA. The indirect whole cell ELISA is similar to the standard ELISA with the addition of an extra antibody step. Thereby the number of binding sites available for the enzyme linked antibody is increased

leading to greater sensitivity of the assay. A dot-blot ELISA, utilising MAbs for the detection of serogroup B meningococcal antigens was developed[54] and can be used for the detection of antigen specific IgA, IgM and IgG in patient sera[21]. This method, using a nitrocellulose membrane in place of a microtitre plate has several advantages over the conventional ELISA. These include, easy to use method; minute amount of antigen required; the white background of the cellulose membrane aides the detection of positive results; measurement of IgG, IgM and IgA antibodies without the need for spectrophotometric readings; and results visible with the naked eye[54,21].

1.7.2.1 Meningococcal Antigen Typing System

MLST or immunotyping of surface-expressed proteins such as PorA, can be used to show high genetic diversity amongst meningococcal populations[185,149]. However, due to the high rates of recombination affecting the meningococcal chromosome, these methods do not take into account additional surface proteins such as those included in the Bexsero® vaccine[19], which may vary in sequence and level of expression[77]. To evaluate the effectiveness of vaccines such as Bexsero® using the accepted correlate of protection against meningococcal disease, would require the SBA assay to be performed against many diverse strains for each geographic region[77]. As for the standard ELISA, this approach is impractical both in terms of serum volume required, particularly in the case of infants, and labour intensity. The meningococcal antigen typing scheme (MATS) was developed as a rapid, reliable system to determine the presence, the diversity, and expression level of antigens included in the Bexsero® vaccine, and to predict the potential coverage of by the vaccine of the circulating isolates[77,294]. MATS is a series of assays that combine conventional PorA genotyping with a unique vaccine antigen-specific ELISA to account for both the extent of immunologic recognition and varying expression of fHbp, NadA, and NHBA among strains of *N. meningitidis*, two variables that help to determine whether bacteria are killed in the SBA assay[77]. As previously shown, ELISA has good correlation with killing of strains by SBA, and is used to measure the quantity and immunologic cross-reactivity of the vaccine antigens[77]. In addition to predicting coverage of the Bexsero® vaccine in different geographical regions, MATS

may also be used for the epidemiological surveillance of meningococci and adapted for the assessment of other pathogens[236].

1.7.3 Multiplex flow cytometric immunoassay

Fluorescence particle based multiplex immunoassays have been developed, using distinctly labelled fluorescent microspheres as carriers for different antigens allowing for the detection of multiple analytes within a single sample[235,230,167,168,193,67]. This technique is based on the ELISA and as such incorporates all the advantage of ELISA with the additional benefits of increased sample throughput and reduced serum volume required[167]. Multiplex assays, such as the Luminex assay (Luminex, Austin, TX, USA) have the additional benefit of optimal antigen presentation through protein coupling protocols, and the use of his-tags etc, for ensuring correct exposure of epitopes when utilising microspheres[230]. A major advantage of a multiplex assay over the conventional ELISA is adaptability, with the potential for 100 analytes to be measured simultaneously. As for the ELISA, these assays are used for the quantification of total serum IgG antibodies, not for functional bactericidal antibodies.

Use of the standard ELISA has been limited with the development of multivalent vaccines such as HexaMen and Nonamen, due to the need for a separate ELISA to quantify IgG responses to each PorA serosubtype. Development of a fluorescence based multiplex assay for the detection of antibodies to specific meningococcal PorA antigens would have several advantages over the standard ELISA method, overcoming many of the limitations of the standard ELISA in the evaluation of multivalent vaccines. With the potential for additional PorA serosubtypes to be included as required, this assay may also be of use in disease and carriage studies for the simultaneous detection of sera responses to multiple PorA serosubtypes. With the development of multicomponent vaccines, the flexibility of the assay to incorporate other antigens, such as PorB, FetA is also of advantage.

1.8 Aims of the Project

The aims and objectives of this thesis were to:

- Produce a set of highly purified meningococcal protein antigens, including seven serosubtypes of PorA, three serotypes of PorB and five variants of FetA;
- Develop a reproducible and reliable multiplex assay to measure antibody responses to specific serosubtypes of PorA;
- Develop a multiplex assay to measure antibody responses in the pre-clinical and clinical trials using a range of sera from individuals vaccinated with one of four meningococcal OMV vaccines;
- Utilise the multiplex assay to evaluate the effect of meningococcal carriage on host immunity using sera collected from healthy individuals as part of two separate carriage studies.

Chapter 2. Methods and Materials

2.1 Materials

All chemicals and reagents were obtained from either Sigma-Aldrich Chemicals (GIllingham, Dorset, UK) or BDH (AnalaR Grade, Poole, UK), unless otherwise stated. The ethylenediaminetetra-acetic acid (EDTA) used was the disodium salt. All water used was purified using a Milli-Q purification system (Millipore, Hertfordshire, UK).

2.1.1 Serum samples

Table 2.1 Source of sera used in this study.

Source	Study	Sera Samples
SEU[2]/VEU[3] (Manchester)	NZ (MNB2) NW (MNB3) Seroepidemiology	9 paired samples 17 paired samples 500 samples
RIVM[4]	Hexamen Nonamen	32 murine sera 10 human samples, (1 pre, 2 post, 7 boost) 32 murine sera
University of Leicester	Carriage	8 samples (new carriers) 5 samples (non-carriers) 10 samples (persistent carriers) 11 samples (cleared carriage)
NIBSC[5]	Unvaccinated serum sample	1 sample (Cat No. 03/118)

Sera used in this study were obtained from several sources as listed in Table 2.1. The HPA Seroepidmiology Unit (SEU) serves as a central resource for the collection of anonymised sera submitted for microbiological and diagnostic testing. Over 150,000 samples have been collected

[2] Seroepidemiology Unit, Health Protection Agency, Manchester, UK.
[3] Vaccine Evaluation Unit, HPA, Manchester, UK.
[4] National Institute for Public Health and the Environment, The Netherlands.
[5] National Institute for Biological Standards and Controls, Hertfordshire, UK.

through collaboration with participating laboratories throughout England and Wales since 1986 and collections are continuing to the present day. These samples form a valuable and unique public health resource. Part of the HPA SEU, is the Vaccine Evaluation Unit (VEU) specialises in the serological determination of immune responses, and has been leading on the evaluation and determination of potential coverage of meningococcal group B vaccines. Meningococcal, pneumococcal, Hib, Diphtheria and Tetanus serology assays developed at the VEU are also widely used for vaccine trials and clinicians (www.hpa.org.uk). The National Vaccine Evaluation Consortium (NVEC) is a consortium that has been working together for over 20 years on vaccine trials bringing together the HPA and academia. MNB2 and MNB3 were NVEC studies, performed in collaboration with manufacturers of the meningitis B vaccine, to assess how the MeNZB and MenBvac vaccines worked in the UK population[92]. Vaccines were administered to healthy laboratory workers on a three dose schedule, with blood and salivary samples obtained prior to vaccination and six weeks after each dose of the vaccine. Samples used in this thesis include pre-- and post-vaccination sera collected six weeks after the third dose of the vaccine.

2.1.2 Solutions

Commonly used buffers, culture media and bacterial growth plates and their formulations are given in Table 2.2 and Table 2.3. All solutions were made up in water purified using a Milli-Q purification system.

Table 2.2 Composition of commonly used buffers. Solutions were made up in water purified using the Milli-Q water purification system.

Name	Formulation
TE	10 mM Tris 1 mM EDTA pH 8.0 adjusted with Hydrochloric acid (HCl)
TlowE	10 mM Tris 0.1 mM EDTA pH 8.0 adjusted with HCl
TAE	40 mM Tris 1 mM EDTA 0.1 % (v/v) Glacial acetic acid
Tris buffered Saline (TBS)	137 mM Sodium chloride (NaCl) 20 mM Tris pH 7.4 adjusted with HCl
Phosphate buffered Saline (PBS)	137 mM NaCl 2.7 mM Potassium chloride (KCl) 10 mM Disodium hydrogen phosphate 1.8 mM Potassium dihydrogen phosphate pH 7.4 adjusted with HCl
Dialysis Buffer (1)	20 mM Tris 0.5 M NaCl 0.05 % (w/v) ZWITTERGENT® 3-14 (Z3-14) pH 7.9 adjusted with Sodium hydroxide (NaOH)
Dialysis Buffer (2)	10 mM Tris 150 mM NaCl 0.05 % (v/v) Triton X-100 pH 7.9 adjusted with NaOH
SDS Running Buffer	0.1 M Tris 0.4 M Glycine 1 % (w/v) Sodium dodecyl sulphate (SDS)

Name	Formulation
Coomassie Brilliant Blue Stain	0.5 % (w/v) Coomassie Brilliant Blue 45 % (v/v) Methanol 10 % (v/v) Glacial acetic acid
Coomassie Brilliant Blue Destain	45 % (v/v) Methanol 10 % (v/v) Glacial acetic acid

Table 2.3 Commonly used broths and growth media. Solutions were made up in water purified using the Milli-Q purification system.

Name	Formulation
Luria Broth (LB)/Agar	10 g/L Tryptone (Oxoid) 8 g/L NaCl 5 g/L Yeast extract (Oxoid) pH 7.5 adjusted with NaOH For agar: 15 g/L Agar flakes
LBKan	Luria broth 30 µg/ml Kanamycin (Kan)
Mueller Hinton agar (Oxoid)	300 g/L Dehydrated beef infusion 17.5 g/L Caesin hydrolysate 1.5 g/L Starch 17 g/L Agar
Blood Agar Base No 2 plates (Oxoid)	15 g/L Proteose peptone 2.5 g/L Liver digest 5 g/L Yeast extract 5 g/L NaCl 12 g/L Agar 7 % Defibrinated horse blood (Oxoid)
Super Optimal broth (SOC) (Novagen, MERCK, Nottingham, UK)	0.5% (w/v) Yeast Extract (Oxoid) 2% (w/v) Tryptone 10 mM NaCl 2.5 mM KCl 10 mM Magnesium chloride 10 mM Magnesium sulphate 20 mM Glucose

2.2 Microbiological techniques

Microbial techniques were performed in a Class I safety cabinet unless otherwise stated.

2.2.1 Propagation of *N. meningitidis* strains

N. meningitidis strains were maintained as frozen stocks at -70 °C on Protect Beads (Technical Services consultants, Lancashire, England). To revive frozen stocks, a single bead was extracted from the vial, and used to inoculate a blood agar base No.2 plate (Oxoid). Following inoculation, plates were incubated at 37 °C in an atmosphere containing 5 % (v/v) carbon dioxide for approximately 18 hours (overnight). A loopful of the resulting culture was then used to inoculate a second plate and ensured a pure culture was obtained. Routine culture of *N. meningitidis* strains was performed on Mueller Hinton agar plates.

2.2.2 Propagation and Maintenance of *E. coli* strains

E. coli strains were maintained on Protect Beads, stored frozen at -70 °C. For the production of frozen stocks, single colonies were selected and inoculated onto agar plates consisting of LB supplemented with 30 µg/ml kanamycin (LBKan). A loopful of the resulting culture was resuspended into the Protect Beads cryo-protectant media following the manufacturer's instructions, and incubated at room temperature for five minutes. The medium was drained and the Protect Beads stored at -70 °C.

E. coli strains were revived, using a single Protect Bead, on LBKan agar plates and streak plates were incubated at 37 °C for approximately 15 hours (overnight). LBKan agar plates were used for the routine culture of *E. coli* strains. Liquid cultures were grown in LBKan broth with overnight incubation at 37 °C in a shaking incubator.

2.3 Molecular Biology Techniques

2.3.1 Preparation of Genomic DNA Promega Wizard® Genomic DNA Purification Kit

Genomic DNA (gDNA) was isolated from each bacterial isolate using the commercially available Wizard® Genomic DNA purification kit (Promega, Wisconsin, UK). The method used was as according to the manufacturers' instructions. Cells harvested from an overnight culture of *N.meningitidis* by centrifugation of 1 ml culture at ≥13,000 x g on an Eppendorf 5412 benchtop centrifuge were resuspended in 600 µL of nuclei lysis solution and incubated at 80 ˚C for 5 mins. A volume of 3 µL of RNase solution (4 mg/ml) was added to the room temperature cell lysate, mixed by gentle inversion and incubated at 37 ˚C for 15 - 60 mins. A volume of 200 µL of protein precipitation solution was added to the cell lysate, vortexed at high speed and incubated on ice for 5 minutes prior to centrifugation at 13,000 x g for 3 mins. Gentle inversion was used to mix the supernatant with 600 µL isopropanol in a fresh tube and the mixture was centrifuged at 13,000 x g for 2 mins on a benchtop centrifuge. The pellet was washed with 600 µL 70 % (v/v) ethanol and left to air-dry for 60 mins prior to rehydration with the addition of 100 µL DNA resuspension solution and storage at -70 ˚C.

2.3.2 Preparation of Plasmid DNA

Plasmid DNA was isolated from each bacterial culture using the commercially available GenElute™ Plasmid Miniprep Kit (Sigma). The method used was as according to the manufacturers' instructions. Cells were harvested from an overnight culture of recombinant *E. coli* culture by centrifugation of 1.5 ml culture at ≥13,000 x g on a benchtop centrifuge. Cell pellets were resuspended in 200 µL resuspension solution, and lysed with the addition of 200 µL lysis solution and incubated at room temperature for 5 mins. Cell debris was precipitated with the addition of 350 µL neutralisation/binding solution, and centrifugation at max speed (13,000 x g) for 10 mins. A column was prepared and washed through with 500 µL column preparation solution, prior to the addition of the cleared lysate and centrifugation at max speed for 1 min. The flow through was discarded and bound DNA was washed using 750 µL diluted wash solution. The column was

centrifuged at max speed for 30 – 60 secs, and the flow through was discarded. Excess ethanol was removed by centrifugation of the dry columns for 1 min at max speed. The column was transferred to a fresh tube and DNA was eluted with the addition of 50 µL elution solution and centrifugation at max speed for 1 minute. Plasmid DNA was stored at -20 °C.

2.3.3 Polymerase Chain Reaction

In accordance with the quantities shown in Table 2.4, the region encoding either the mature PorA, PorB, FetA, or fHbp antigen was amplified from the meningococcal DNA preparation using the polymerase chain reaction (PCR). Oligonucleotide primers (Invitrogen, Paisley, UK) as shown in Table 2.5 were used to amplify the genes. Primers were designed with 5' sequence extensions required for ligation into the vector (as underlined), followed by the conserved start sequence of the target gene, as identified using DNA sequence alignment. All genes were cloned without the signal peptide in order to aid in purification of the expressed protein. Where multiple PCRs were set up for the same antigen, a master mix of all the components with the exception of the template was prepared and dispensed into individual tubes. A negative control was included using sterile water in place of template DNA to detect any contamination of the reagents. The PCR reactions were carried out in 0.2 ml thin walled tubes in the DNA Engine thermocycler (MJ Research), DNA Engine Dyad thermocycler (Bio-Rad, Hemel Hempstead, UK), or the Veriti 96 well thermal cycler (Roche, Hemel Hempstead, UK), programmed as shown in Table 2.6.

At the beginning of each reaction the temperature was held at 94 °C for 5 mins. At the end of the reaction, the temperature was held at 72 °C for 10 mins, and finally kept at 4 °C until tubes were removed from the block. Several methods were used to optimise PCR conditions. These included the use of a gradient PCR machine to assay a range of extension temperature for the reaction, the addition of Magnesium Chloride in the reaction mix, and the use of sero- and serosubtype specific primers where the target gene did not initially amplify.

Table 2.4 Reagents used in the standard Ek/LIC insert PCR

Component (Roche)	Antigen			
	porA	***porB***	***fetA***	***fHbp***
Forward Primer	1 µM	1 µM	1 µM	0.5 µM
Reverse Primer	1 µM	1 µM	1 µM	0.5 µM
PCR Reaction Buffer	1.5 mM	1.5 mM	1.5 mM	1.5 mM
Magnesium Chloride	-	1.5 mM	-	-
Deoxynucleotide Triphosphate (dNTP)	100 µM	100 µM	100 µM	100 µM
AmpliTaq polymerase	50 mU/µL	50 mU/µL	50 mU/µL	50 mU/µL
gDna	2 µL	2 µL	2 µL	2 µL

Table 2.5 Sequence of oligonucleotide primers used for PCR amplification of the *porA*, *porB*, *fetA* and *fHbp* genes.

Gene	Primer Name	Orientation	Primer Sequence
porA	PorALICF	Forward	GACGACGACAAGATCGATGTCAGCCTATACGGC
	PorALICR2	Reverse	GAGGAGAAGCCCGGTCTAGAATTTGTGGCGCAAAC
	PorALICF5,2	Forward	GACGACGACAAGATCGATGTTAGCCTGTACGGC
porB	PorBLICF	Forward	GACGACGACAAGATTGACGTTACCCTGTACGG
	PorBLICR	Reverse	GAGGAGAAGCCCGGTCTAGAATTTGTGGCGCAG
	PorBLICF2b	Forward	GACGACGACAAGATTGACGTTACCTTGTACGG
fetA	FetALICF	Forward	GACGACGACAAGATGGCAGAAAATAATGCCAAGGTCG
	FetALICR2	Reverse	GAGGAGAAGCCCGGTCTAGAACTTGTAGTTCACGC
fHbp	fhbpEKLICf1	Forward	GACGACGACAAGATGTGCAGCAGCGGA
	fhbpEKLICr1	Reverse	GAGGAGAAGCCCGGTATTGCTTGGCGGCAAG
	fhbpEKLICr2	Reverse	GAGGAGAAGCCCGGTACTGCTTGGCGGCAAG
	fhbpEKLICr3	Reverse	GAGGAGAAGCCCGGTACTGTTTGCCGGCGAT

Table 2.6 PCR programmes used for amplification of the *porA*, *porB*, *fetA* and *fHbp* genes.

Stage	Time (secs)	Temperature		
		porA / fHbp	***porB***	***feta***
Denaturation	60	94 °C	94 °C	94 °C
Annealing	60	65 °C	60 °C	63 °C
Extension	120	72 °C	72 °C	72 °C
For 30 cycles				

2.3.4 Agarose gel electrophoresis

Amplification products from the PCR and DNA extraction protocols were analysed using agarose gel electrophoresis. 0.8 % (w/v) agarose (Gibco®, Fisher, Leicestershire, UK) was heat dissolved in 1 x TAE buffer and allowed to cool before SafeView (NBS Biologicals, Cambridgeshire, UK) was added to the gel mixture (5 µL/100 ml). The gel was cast and allowed to set at room temperature for approximately 1 hour. The gel was submerged in 1 x TAE buffer, DNA samples were mixed with gel loading buffer (6 x concentration: 0.25 % (w/v) bromophenol blue, 40 % (w/v) sucrose in TE buffer), and the samples were loaded into individual wells using a pipette. A 100 bp / 1 Kb DNA ladder (Invitrogen) was run alongside the samples to allow estimation of DNA fragment size.

Following electrophoresis at 80 V for approximately 1 hour, DNA fragments were visualised using an ultra-violet (UV) trans-illuminator, photographed using the Kodak MI camera and fragment size was estimated using the Kodak MI analysis software.

2.3.5 Purification of PCR products

PCR amplified DNA was purified using poly ethylene glycol (PEG) precipitation prior to use in the cloning protocol. A volume of 95 µL of the PCR product was mixed with 60 µL of 20 % (v/v) PEG_{8000} / 2.5 M NaCl and incubated at 37 °C for 10 mins. The pellet was harvested by centrifugation at full speed for 10 minutes, and washed with 500 µL of 80 % (v/v) ethanol. The air-dried pellet was resuspended in 25 µL TlowE buffer and stored at -20 °C.

2.3.6 Cloning of *porA, porB and fetA* genes into pET expression vector (Novagen®)

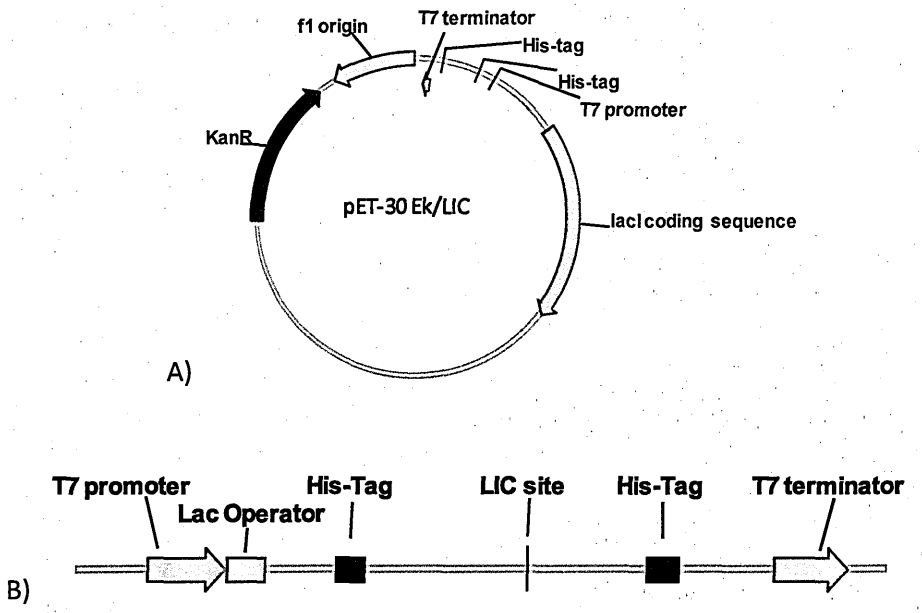


Figure 2.1 pET-30 Ek/LIC vector map. A) shows the complete vector, whilst b) only shows the cloning/expression region. His-tags are present on either side of the Ligation Independent Cloning (LIC) site to ensure the required gene is expressed with a His-tag. The N-terminal his-tag is used in this study and is achieved using the stop codon at the end of the cloned gene sequence. Restriction sites have not been shown in these maps.

The pET cloning and expression system was developed to facilitate directional cloning of PCR products allowing for the incorporation of a polyhistidine tag at either the N' or C' terminus which can be used to aid protein purification. Protein purification is further facilitated by the production of inclusion bodies, formed as a consequence of high protein expression in the absence of a signal peptide. The use of this cloning system and the resulting inclusion bodies allowed expressed proteins to be easily isolated by centrifugation, with the addition of a His-tag used for purification purposes and also for bead conjugation thereby ensuring high yield and purity. The presence of a gene coding antibiotic resistance aids in the selection process with only cells that have incorporated the vector capable of growth on selection media.

Annealing of the vector and the insert was performed utilising T4 DNA polymerase to create specific single strand overhangs in the vector and the insert allowing for more efficient annealing

without the need for restriction digestion or ligation. The insert was controlled by a T7 promoter, which was in turn under the control of a *lac* promoter and operator allowing for inducible expression of the target protein as required. The presence of a *lacI* coding sequence in the vector results in the production of lac repressor which binds to the *lac* operator and, in turn ensures that T7 RNA polymerase is not able to bind to the T7 promotor and target protein can not be expressed. Expression cells such as BL21 (DE3) have a *lac* promoter sequence in front of the *lac* operator that native *E.coli* RNA polymerase is able to bind, in place of a T7 promotor sequence. Following induction, the lac repressor is bound by isopropyl β-D-1-thiogalactopyranoside (IPTG), native RNA polymerase is able to bind to the *lac* promoter and T7 RNA polymerase can be expressed. *E.coli* T7 RNA polymerase binds to the T7 promotor on the pET30 vector, and the target gene is expressed. IPTG is used as inducer of the *lac operon* as it is not involved in any metabolic pathways, and is not broken down or used by the cell and therefore remains constant throughout the induction process. Cloning into NovaBlue was used for screening colonies and for the establishment of the plasmid. Novablue cells do not possess a T7 RNA polymerase gene and therefore ensure plasmid stability due to a lack of protein expression. Cloning of the vector into BL21(DE3) cells is used for expression of the target protein.

For this study, amplified DNA fragments were annealed into the pET-30 Ek/LIC vector, transformed initially into NovaBlue competent cells, and later into the expression host cells BL21 (DE3) expression cells using the same method. Cells containing the vector and the desired DNA fragment were selected using a kanamycin resistance gene. Purified PCR products were treated with T4 DNA polymerase in order to produce the compatible single stranded overhangs required for cloning. All components used were supplied as a kit (Novagen). Individual components were compiled, as shown in Table 2.7, in a fresh tube stored on ice. The reagents were mixed with a pipette tip and incubated at 22 °C for 30 min. The reaction was incubated at 75 °C for 20 mins to deactivate the enzyme and stop the reaction. The treated product was then stored at -20 °C.

Table 2.7 Final concentration of reagents used in T4 DNA polymerase treatment

Component (Novagen)	Concentration
Purified PCR product	~0.01 µM
T4 DNA polymerase buffer	1 x
2'-Deoxyadenosine-5'-Triphosphate (dATP)	2.5 mM
Dithiothreitol (DTT)	5 mM
T4 DNA Polymerase	50 mU/µL

The T4 DNA treated PCR product was annealed to the pET-30 Ek/LIC vector (Novagen) prior to the transformation into competent cells. For each insert, 1 µL of Ek/LIC pET-30 Vector was mixed with 2 µL of the T4 DNA polymerase treated Ek/LIC insert. The mix was stirred with a pipette tip and incubated at 22 °C for 5 mins, before 1 µL of 25 mM EDTA was added. The mixture was again stirred using a pipette tip and incubated at 22 °C for 5 mins. The annealed vector/insert was stored at -20 °C.

NovaBlue GigaSingles™ competent cells (Novagen) and BL21(DE3) competent cells (Novagen) were used as detailed in the Novagen Ek/LIC vector kit protocol. The method was performed according to the manufacturers' instructions with a single tube of competent cells used per transformation. Competent cells were thawed on ice for 5 mins and then flicked to ensure complete and even resuspension of the cells. 1 µL of the annealing mixture was added to the cells and stirred using the pipette tip before the mixture was incubated on ice for 5 mins. Each tube was then heated for exactly 30 secs at 42 °C (without shaking) and incubated on ice for a further 2 mins. A volume of 250 µL of room temperature SOC media (Novagen) was added to each tube, and incubated with shaking for 60 mins at 37 °C. Transformed cells were plated on to LBKan agar plates inverted and incubated at 37 °C overnight. Several volumes (5 µL, 20 µL and 50 µL) of cell solution were used to inoculate each plate in order to ensure individual colonies were produced.

In accordance with the quantities shown in Table 2.8, the region encoding the incorporated DNA insert was amplified from each transformed colony using PCR. The T7 primer binding site was

found on either side of the insertion site, and so can be used to estimate the size of the incorporated DNA insert. The sequence of the T7 promoter and terminator primers can be found in Table 2.11. Where multiple PCR reactions were set up, a master mix of all the components with the exception of the template was prepared and dispensed into individual tubes. A negative control was included using sterile water in place of transformed cells to detect any contamination of the reagents. The PCR reactions were carried out in 0.2 ml thin walled tubes placed in the DNA Engine (MJ Research), DNA Engine Dyad (Bio-Rad), or the Veriti 96 well thermal cycler (Roche), programmed as shown in Table 2.9. At the beginning of each reaction the temperature was held at 94 °C for 5 mins. At the end of the reaction, the temperature was held at 72 °C for 10 mins, and finally kept at 4 °C until tubes were removed from the block.

The resulting PCR product was examined by gel electrophoresis, UV visualisation and fragment analysis to estimate the size of the DNA insert. Colonies containing a single DNA fragment of the expected size were taken forward for plasmid DNA extraction and nucleotide sequencing.

Table 2.8 Final Concentration of reagents used in the Colony Screening PCR.

Component (Roche)	Concentration
T7 Promoter Primer	1 µM
T7 Terminator Primer	1 µM
PCR Reaction Buffer	1.5 mM
dNTP	200 µM
AmpliTaq polymerase	50 mU/µL

Table 2.9 PCR programmes used for amplification of the Incorporated DNA insert

Stage	Temperature	Time (seconds)
Denaturation	94 °C	60
Annealing	43 °C	60
Extension	72 °C	120
For 30 cycles		

2.3.7 Nucleotide sequencing and sequence analysis

ABI prism™ BigDye™ Terminator Cycle Sequencing Ready reaction kit (Roche) was used in the preparation of all sequence reactions. Reactions were set up as shown in Table 2.10 below. The sequence of the oligonucleotides used in the sequencing reactions are given in Table 2.11. All inserts were sequenced using the T7 promoter and terminator regions on the vector in addition to primers targeted to internal portions of the gene of interest. PorA was sequenced using the 8U and 122L sequencing primers described previously[290]. DNA alignments were produced for each gene of interest, and conserved regions were used to design primers in order to allow reading of the complete gene sequence. The T7 promoter and terminator regions were sequenced using primers recommended by Novagen for use with the pET30 Ek/LIC vector kits.

Table 2.10 Reagents used in the standard sequencing reaction.

Reagent	Concentration
Terminator Ready Reaction Mix	1 x
PCR product (DNA template)	~ 20 ng
Sequencing Buffer	1 x
Sequencing Primer	0.5 mM

Cycle sequencing of the sequencing reactions was performed according to the conditions stated in Table 2.12 and run on a DNA Engine (MJ Research) following pre-existing laboratory protocols. All centrifugation steps for this method were performed using the Sorvall Super T21 centrifuge, with the ST-H750 rotor. Products from the sequencing PCR were purified using ethanol precipitation. A volume of 10 µL of sterile water and 5 µL 125 mM EDTA were added to each sequencing reaction, together with 80 µL 99 % (v/v) ethanol and each sample was incubated at room temperature for approximately 1 hour. DNA was collected by centrifugation at 3000 x g, washed with 200 µL 70 % (v/v) ethanol and incubated at room temperature for 5 mins. DNA was harvested by centrifugation at 1800 x g for 15 mins and the pellet was dried by spinning the open tubes

inverted in the centrifuge for 2 mins at 900 x g. Each pellet was resuspended in 10 µL Hi-Dye Formamide (Roche) and stored at -20°C prior to loading on the sequencer.

Table 2.11 Sequence of oligonucleotide primers used for sequencing pET-30 vector inserts.

Gene	Primer Name	Orientation	Primer Sequence
pET vector	T7 promoter	Forward	TAATACGACTCACTATAGGG
	T7 terminator	Reverse	GCTAGTTATTGCTCAGCGG
porA	8U	Forward	TCCGTACGCTACGATTCTCC
	122L	Reverse	GGCGAGATTCAAGCCGCC
porB	PorBfwd	Forward	GCGAAGCTTCCCTGATTGCCCTGACTTTG
	PorBrev	Reverse	GCGAAGCTTTGTGGCGCAGACCGAC
fetA	S1	Forward	CGGCGCAAGCGTATTCGG
	S4	Reverse	GCGCTTTGATTTCCTGATGG
fHbp	fHbpf1	Forward	GTCGCCGCCGACATCGGTGCGG
	fHbpr2	Reverse	CTGCTTGGCGGCGAAATC
	fHbpsr3	Reverse	GGAACTCTCCACTCTCCAAGG

Table 2.12 Sequencing programme used for cycle sequencing reactions

Temperature (°C)	Time (seconds)
96	30
50	15
60	240
For 25 cycles	

Separation and detection of the extension products, for all the genes sequenced, was carried out on an ABI Prism 3130xl Genetic Analyzer (Roche). Sequences were analysed using either the Vector NTI software analysis programme (Invitrogen), or the Ridom Trace Edit Pro (Ridom GmbH, Germany). Variants of *PorA, FetA* and *fHbp* were identified using sequences published on the Pubmlst.org website (http://pubmlst.org/*Neisseria*/).

2.4 Biochemistry Techniques

2.4.1 Expression of target proteins using the His-Bind System (Novagen)

An overnight culture of LBKan and 1 % (w/v) glucose, inoculated with a single bacterial colony was used to seed 100 ml of LBKan and 1 % (w/v) glucose in a 500 ml flask. The volume of overnight culture used was sufficient to give an optical density as measured at an Optical Density wavelength of 600 nm (OD600) value of 0.05, measured using a WPA Biowave Cell Density meter (Biochrom WPA, Cambridge, UK). This is a widely used value in protein expression protocols and ensured the bacterial cells were in the optimal growth phase prior to induction with IPTG.

The culture was incubated at 37 °C with shaking and sampled hourly until the OD600 value was in the range of 0.5 and 0.6. This value is again widely used in protein expression protocols and is when the cells have reached the mid log phase of growth and ensured protein production following induction.

Bacterial cultures were induced with 1 mM IPTG, and incubated with shaking at 37 °C. Whole cell lysate (WCL) samples were taken every hour and the OD600 value was recorded and used to prepare a growth curve. Following protein induction, the culture was chilled on ice for 5 mins and cells were collected by centrifugation at 4000 x g (Sorvall RT, or RC-5B for larger cultures) for 20 mins. The culture supernatant was discarded and the wet weight of the pellet was calculated. The cell pellet was stored at -70 °C.

2.4.2 Protein Purification

Protein purification was performed using the Bugbuster® protein extraction reagent (Novagen) following the manufacturers protocols. Bugbuster is a propriety mixture of non-ionic detergents, used to perforate the cell wall without denaturing soluble protein. The addition of rLysozyme™ solution and Lysonase™ bioprocessing reagent (all supplied by Novagen) increased the efficiency of protein extraction and facilitated the downstream processing of protein extracts. Lysonase bioprocessing reagent was an optimised mixture of rLysozyme and benzonase nuclease.

rLysozyme was used to lyse gram-negative bacteria whilst benzonase nuclease was an endonuclease used to degrade all forms of DNA and RNA, resulting in reduced extract viscosity and increased protein yield. All centrifugation steps for this method were performed using the Sorvall RT centrifuge. A pre-weighed centrifuge tube was used for the collection of the pellet at each stage of purification, in order to allow measurement of the resulting pellet.

The frozen cell pellet was thawed, and resuspended in room temperature BugBuster® reagent using 5 ml of Bugbuster® reagent for every gram of wet cell paste. Lysonase was added at 10 µL per gram of wet cell paste, and the cell suspension was mixed on a shaking platform at a slow setting until it was no longer viscous (approximately 20 mins). The suspension was centrifuged for 20 mins at 16,000 x g and 4 ˚C and the pellet was resuspended in BugBuster® reagent (5 ml/gram pellet wet weight). rLysozyme was added to a final concentration of 1 KU/ml and the mixture was incubated at room temperature for 5 mins. A further 6 volumes of 1:10 diluted BugBuster® reagent (in deionised water) was added and inclusion bodies were harvested by centrifugation at 5,000 x g for 15 mins at 4 ˚C.

The pellet containing the inclusion bodies was washed twice by resuspension in half the original volume of 1:10 diluted BugBuster® reagent (10 ml per gram of pellet wet weight), and centrifuged at 5,000 x g for 15 mins at 4 ˚C. Purified inclusion bodies were collected by centrifugation at 16,000 x g for 15 mins at 4 ˚C, and resolubilised in TE + 8 M Urea to give a final concentration of 15 mg wet weight per ml of buffer. An equal volume of 20 mM Tris, 1 M NaCl, 2 % (w/v) Z3-14 was added to the resolubilised solution in order to give the supernatant a final concentration of 15 mM Tris, 4 M Urea, 0.5 % (w/v) NaCl, 1 % (w/v) Z3-14 prior to dialysis.

Protein was refolded by dialysis against Dialysis Buffer 1. The supernatant was transferred to dialysis tubing (Pierce, Fisher), the air bubbles removed and the tubes sealed using clips. Each tube was placed in a beaker containing 25 x volume Dialysis Buffer 1 and incubated with gentle stirring for 2-3 hours at room temperature. Five changes of buffer were used with the final change incubated at 4 ˚C overnight. Debris was removed by centrifugation at 16,000 x g for 10

mins at 4 °C and refolded protein was collected. This was a pre-existing method used in the laboratory previously for purification of PorA and FetA, and refolding of proteins using this protocol have previously been determined. Due to the use of Triton X-100 traditional methods for determination of protein refolding could not be used in this study. For the purposes of this study, confirmation of refolding and the correct presentation of the variable epitopes of PorA and PorB were established with the use of monoclonal antibodies. Anti-FetA antibodies were not available, and so could not be used for the structural confirmation.

The following buffers were provided as concentrated solutions in the Novagen His-Bind kit and were diluted and supplemented with either Z3-14 or Triton X-100 as described. The volume of resin was calculated allowing 1 ml of resin for every 8 mg of protein to be purified.

- Charge buffer (50 mM Nickel sulphate ($NiSO_4$) (Sigma))
- Bind buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 5 mM Imidazole, 0.05 % (w/v) Z3-14)
- Wash buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 60 mM Imidazole, (9 mM for FetA) 0.2 % (v/v) Triton X-100)
- Elute Buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 1 M Imidazole, 0.2 % (v/v) Triton X-100)

For small scale purification, columns were packed with 2 ml his-bind resin (Novagen) using a wide mouthed pipette and allowed to settle under gravity flow, to give a 1 ml settled bed volume. Once the level of storage buffer dropped to the top of the column bed, the column was washed and charged with 3 column volumes of water, 5 column volumes of 1 x charge buffer and 3 column volumes of 1 x bind buffer. For large scale purification, 20 ml of resin was used, allowing for a 10 ml settled bed volume, and each step was performed using centrifugation at 500 x g (Sorvall RT centrifuge) for 1 minute.

Imidazole was added to the refolded protein extract at a final concentration of 5 mM before the extract was loaded onto the column. The extract flow through was collected and reapplied to the column to ensure sufficient binding of the protein to the charged resin. The column was washed with 10 column volumes 1 x binding buffer and 6 column volumes 1 x wash buffer prior to

elution of the purified protein using 6 column volumes of 1 x elution buffer. Imidazole was removed from the purified protein using dialysis against Dialysis Buffer 2. Imidazole acts by competing with Nickel sulphate to bind the His-tag on the target protein. Low concentrations of Imidazole are used in the wash buffer to limit non-specific binding, whilst a much higher concentration in the elution buffer ensures elution of the protein from the Nickel charged protein purification column.

Protein was concentrated using Ultra-15 centrifugal filters (Amicon, Millipore, Billerica, MA, USA) with a 10 kDa molecular weight cut off and centrifugation at 4000 x g for 20 mins (Sorvall RT).

2.4.3 Protein Concentration

SDS polyacrylamide gel electrophoresis (PAGE) was used to separate proteins according to size. Gel casting equipment (Bio-Rad) was assembled and the resolving gel was prepared as shown in Table 2.13. The gel was poured between the assembled plates, and overlaid with water saturated butanol to ensure a flat even surface at the top of the gel, and to exclude air which would have prevented polymerisation. The gel was allowed to set for an hour at room temperature, prior to removal of the butanol with several washes of water. The sandwich was blotted dry with blotting paper and the resolving gel was overlaid with the stacking gel, assembled as described in

Table 2.14. A comb was inserted into the top of the stacking gel and the gel was allowed to set at room temperature.

Table 2.13 Reagents used in the preparation of the resolving gel.

Reagent	Concentration
Bis-Acrylamide (Bio-Rad)	12 % (v/v)
Tris-HCl pH 8.8	390 mM
SDS	0.1 % (w/v)
Ammonium persulphate (APS) (Amresco)	0.1 % (w/v)
Tetramethylethylenediamine (TEMED) (Bio-Rad)	0.04 % (v/v)
Total Volume	35 ml

Table 2.14 Reagents used in the preparation of the stacking gel.

Reagent	Concentration
Bis-Acrylamide	9.75 % (v/v)
Tris-HCl pH 8.8	325 mM
SDS	0.26 % (w/v)
APS	0.25 % (w/v)
TEMED	0.1 % (v/v)

The gel was submerged in 1 x SDS running buffer and protein samples were mixed with an equal volume of 1 x SDS loading buffer, made up as shown in Table 2.15. The mixture was incubated at 97 °C for 5 mins and loaded into individual wells using a pipette. A prestained SDS-PAGE broad range standard (Bio-Rad) was used to allow estimation of protein band size, following electrophoresis at 30 mA (or 8 mA if run overnight) until the dye front reached the bottom of the gel. In order to visualise the protein bands, the gel was submerged in Coomassie brilliant blue solution and incubated at room temperature overnight, with gentle shaking. Excess stain was removed with several washes in Coomassie brilliant blue destain until bands were clearly visible. The gel was then photographed and band size was estimated using the Kodak MI camera, and Kodak MI analysis software.

Table 2.15 Chemical composition of the SDS loading buffer.

Reagent	Concentration
Sucrose	10 % (w/v)
SDS	2 % (w/v)
Tris-HCl pH 6.8	62.5 mM
B-Mercaptoethanol	5 % (v/v)
Bromophenol Blue	Enough to add colour

Protein concentration was determined using the BCA assay (Perbio, Fisher). A standard curve was produced using nine concentrations of bovine serum albumin (BSA) ranging from 0 – 2 mg/ml in water. The working reagent was prepared by combining 50 parts Reagent A with 1 part Reagent B.

Samples were diluted in dialysis buffer to give a dilution range from 1:10 to 1:100 in order to ensure the concentration could be calculated from the standard curve. A volume of 200 µL of the working reagent was added to 10 µL of each standard and sample (undiluted and diluted) in a 96-well plate, and mixed for 30 secs on a microtitre plate shaker. The plate was covered with a plate lid and incubated at 37 °C for 30 mins. The absorbance was read at a wavelength of 570 nm using a microtitre plate reader (Labsystems Multiskan MS), and used to produce a standard curve. A line of best fit was used to estimate concentration of the purified protein.

2.4.4 Monoclonal Antibody Concentration

Monoclonal antibody concentration was determined using the Easy-Titer Mouse-IgG kit (Thermo, Fisher). A standard curve was produced using six concentrations of Mouse IgG whole molecule control standard (Thermo, Fisher) ranging from 500 – 15.6 ng/ml in dilution buffer. Anti-IgG sensitised beads were mixed thoroughly and vortexed vigorously for 60 seconds prior to use in the assay. MAbs were diluted in dilution buffer to give a dilution range from 1:100 to 1:5000 in order to ensure the concentration could be calculated from the standard curve. A volume of 20 µL of the sensitised beads was added to 20 µL of each standard and sample (undiluted and diluted) in a 96 well plate and mixed for 5 mins on a microtitre plate shaker on moderate to high speed. A volume of 100 µL blocking buffer was added to each well and mixed for 5 mins on a microtitre plate shaker on moderate speed to prevent spills. The absorbance was read at a wavelength of 340 nm using a microtitre plate reader (Labsystems Multiskan MS), and used to produce a standard curve. A line of best fit was used to estimate MAb concentration.

2.5 Liquichip Assay

A Liquichip assay is a microsphere assay based on Luminex xMAP technology and involves the use of internally labelled fluorescent microspheres as a solid phase platform for the interaction of immobilised, microsphere bound capture molecules with analytes in solution. The reaction was quantified using a secondary reporter molecule specific for the analyte. Each microsphere is labelled with a combination of red and infrared dyes, resulting in a unique fluorescent signature,

with the potential for up to 100 different microspheres to be used within a single assay. Coupling different capture molecules to different microspheres enables the individual detection of multiple antigens simultaneously.

2.5.1 Bead Preparation

Bio-Plex™ carboxy microsphere sets (Bio-Rad) were prepared as described previously[170]. Bio-Plex™ carboxy microsphere sets were washed 3 times and resuspended to 2 x 10^8 microspheres/ml in 0.05 M 2-(N-morpholino)ethanesulfonic acid buffer (MES) (pH 6.5, Fisher Scientific) with centrifugation at 14,000 x g for 2 mins in a benchtop centrifuge. Microsphere sets were activated with the addition of 0.1 mg N-hydroxysuccinimide (NHS) (Acros chemicals, Fisher) and 1 mg 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDC) (Fisher) per 1 x 10^8 microspheres and incubated at 22 °C for 10 mins with shaking.

Microspheres were collected by centrifugation at 14,000 x g for 2 mins, and resuspended in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8.0) (Sigma) to give a final concentration of 1 x 10^8 microspheres/ml. 1 mg of EDC and 0.1 mg NHS were added per 1 x 10^8 microspheres prior to the conjugation with N-(5-amino-1-carboxypentyl)iminodiacetic acid (NTA) ligand (Sigma, Fluka chemicals). The microsphere mix was incubated with 4 x 10^{10} molecules of NTA ligand for every microsphere and incubated at 22 °C for 18 hours.

Microspheres were harvested by centrifugation at 14,000 x g for 2 mins and resuspended in 1 ml 50 mM Tris buffer, pH 8.0 + 0.005 % tween 20 in order to remove residual free carboxy groups. The pellet was washed twice with 600 µL PBS + 0.005 % tween 20 using centrifugation at 14,000 x g for 5 mins. Microspheres were resuspended in 1 ml of 500 µM nickel chloride (Fisher) and incubated at 22 °C for 2 hours, prior to 3 washes and final pellet resuspension to 5 x 10^7 microspheres/ml in PBS + 0.005 % (v/v) tween 20. Nickel coated microsphere sets were stored at 4 °C protected from light.

Nickel coated microspheres were diluted to 1 x 10^7 microspheres/ml in PBS/BSA/Triton X 100 buffer (PBS + 0.1 % (w/v) BSA + 0.05 % (v/v) Triton X-100) in preparation for protein coating and

vortexed to ensure an even suspension, prior to incubation with an equal volume of 100 µg/ml purified protein solution. The mixture was protected from light and incubated at 4 °C for 3 days.

Microspheres were transferred to a microcentrifuge tube and harvested by centrifugation at 10,000 x g for 2 mins using a benchtop centrifuge. The pellet was washed twice with 1.5 ml PBS/BSA/Triton X-100 buffer and resuspended at approximately 2 x 10^7 microspheres/ml in PBS/BSA/Triton X-100 + 0.05 % (w/v) sodium azide buffer.

Whilst conformation of the PorA protein following microsphere labelling was not checked, antibodies specific to either VR1 or VR2 regions were used to determine the correct presentation of the antibody binding sites for this protein using the Liquichip assay (chapter 4).

A Guava EasyCyte flow cytometer (Millipore) was used to count the protein labelled microsphere. A sample sheet was set up, using the following parameters: 2500 microsphere counts; 300 sec timeout; 0 replicates; medium flow rate, and microsphere solutions were used to prepare a 1:125 diluted sample in PBS/BSA/Triton X-100 buffer. The samples were read according to the machine settings described in Table 2.16 and the number of microspheres per millilitre was calculated.

Table 2.16 Setting used to set up the Guava EasyCyte flow Cytometer

Variable	Setting
Red	850
Yellow	850
Green	0
FSC Gain	32
SSC	346
MinMax	150 %
Flow Rate	Medium

2.5.2 PorA Assay

A LiquiChip filter microplate (Qiagen, Crawley, UK) was used for the assay, and the solution was removed following each step using a vacuum pump.

Microsphere sets were made up to contain 2500 of each PorA labelled microspheres, in a total volume of 25 µL, for every assay point to be measured. In a separate microplate, six 3 - fold serial dilutions of the standard, the samples and a buffer-only control were prepared, with 25 µL of sera used for each assay point. Each well of the 96-well filter microplate was pre-wet with 50 µL PBS/BSA/Triton X-100 buffer, which was removed using the vacuum pump and discarded. This step ensured proper filtration of the samples. A volume of 25 µL of the serum sample and the standard was added to the plate followed by 25 µL of the microsphere mixture, then vortexed to ensure an even suspension of microspheres. The microplate was agitated with a Whirlimixer (Fisher) used with a plate attachment, ensuring the contents of the wells were not spilt. The plate was covered in foil and incubated on a platform shaker at room temperature for 25 mins. The supernatant was removed using the vacuum pump and the microplate was washed twice with 50 µL PBS/BSA/Triton X-100 buffer, to remove any unbound antibodies.

A total of 200 ng anti-IgG recombinant phycoerythrin (rPE) conjugate (directed against human or mouse IgG as required) in a volume of 50 µL was added to each well and the plate was agitated and incubated as before. The supernatant was removed and the microplate was washed twice with 50 µL PBS/BSA/Triton X-100 buffer to remove any unbound antibodies. The microsphere/serum mixture was resuspended in 150 µL PBS/BSA/Triton X-100 buffer and agitated using a whirlimixer with a plate attachment prior to loading on the Liquichip 100 workstation (Luminex, Qiagen).

A sample volume of 70 µL, a minimum of 100 microspheres and a timeout of 45 secs were used for the measurement of each sample. These values were used as stated in the Liquichip manual. Microsphere regions were matched with the corresponding protein, and standards were assigned an arbitrary concentration of 0 - 10 ng/ml in order to aid calculation of sample concentrations and

to allow comparison of samples over several assays. Due to the unknown concentration of antibodies present in the human standard used, the most concentrated standard (1:100 dilution) was assigned an arbitrary concentration of 10 ng/ml, and the most dilute standard (1:24300 dilution) assigned an arbitrary concentration of 0.04 ng/ml. In total six dilution in a three dilution series were used. These values were then used to plot a standard curve for the determination of sample concentrations.

Prior to use, the Liquichip workstation was calibrated using both control and calibration beads as provided by Qiagen. Using the Luminex IS 2.1 software (Qiagen), a standard curve was plotted using the 4 Parameter Logistic (4PL) nonlinear regression model and used to calculate the arbitrary concentrations for each of sample. This model is commonly used for curve-fitting analysis in bioassays or immunoassays such as ELISAs.

Chapter 3. Cloning, Expression and Purification of the PorA, PorB and FetA gene variants.

3.1 Introduction

The aim of this project was to develop a multiplex assay for the detection and quantification of outer membrane proteins. In order to do this, variants of PorA, PorB and FetA had to be cloned, expressed and purified. These proteins, particularly PorA and PorB, are important as vaccine components[79] as they have been shown to be the targets of bactericidal antibodies[347]. These proteins are also used for serotyping (PorB) and serosubtyping (PorA) meningococci[101].

Stocks of purified antigens needed to be produced in order to develop a multiplex assay for the detection of PorA, PorB and FetA variants. For this: seven PorA serosubtypes; a PorA mutant (P1._,_), cloned and expressed as a PorA protein in which both VR1 and VR2 regions were deleted; four PorB serotypes; and five FetA variants were cloned and used for the expression of protein. Polyhistidine-tags (His-tag) are able to bind metal ions such as nickel with good efficiency under specific buffer conditions. The proteins were expressed with a His-tag at their N' terminus to facilitate affinity purification of protein using a His-Bind resin column charged with a nickel sulphate solution, with proteins bound to the nickel within the column prior to elution with an elution agent such as Imidazole. Additionally, affinity between the His-tag and the nickel ions can be used and as a method for coupling the protein to a nickel coated microsphere for use in the assay described in Chapter 4.

For each of these antigens, variants were chosen for their association with hypervirulent lineages, ability to cause disease and their continued presence among circulating strains in England and Wales[126]. In addition, PorA serosubtypes were chosen to include variants used in vaccines. A PorA P1._,_ mutant was also cloned, and protein from this mutant was purified to determine the responses, if any, to the backbone of the PorA protein. The pET cloning and expression system is widely used for the protein expression was chosen for several reasons. This system allowed for the directional cloning of PCR products without restriction digestion; the incorporation of a

polyhistidine tag (Figure 3.1); and has been shown to be reliable for high efficiency expression and purification of meningococcal proteins[129], *N. gonorrhoeae* proteins NspA[358], and PorB[57,178].

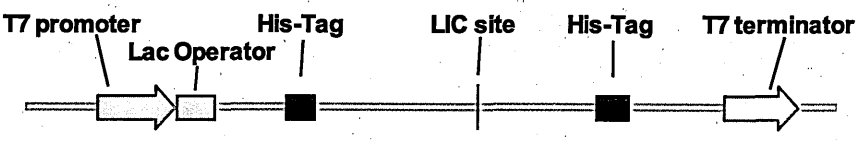


Figure 3.1 Cloning/Expression region of the pET30/EkLIC vector.

In this system, the expression of the cloned gene is under the control of a T7 promoter, which is in turn under the control of a lac promoter and operator allowing for inducible expression of the target protein as required. The selective and active T7 promotor ensure that almost all of the cell's resources are directed to expression of the target gene resulting in the protein of interest making up more than 50 % of the total cell proteins[129]. A kanamycin resistance gene, also contained within the vector, provides for the selection of transformed colonies and adds stability to the vector. The formation of inclusion bodies, a consequence of high protein expression in the absence of a signal peptide, facilitates purification, resulting in less contamination with unwanted *E. coli* proteins. The protein antigens were purified using immobilised metal affinity chromatography (IMAC). The target protein, expressed with an incorporated His-tag, binds to the nickel cations, which in turn bind to the His-Bind resin column. Elution of protein from the His-Bind column was achieved by the use of imidazole containing buffer.

The porin PorA is 44 – 47 KDa outer membrane protein. It has a 16-stranded β-barrel structure with eight cell surface exposed loops, and two distinct VRs, VR1 and VR2 corresponding to loops 1 and 4, respectively[198,188]. VR1 and VR2 are the longest surface exposed loops and induce bactericidal antibodies[324]. A third VR is located on loop 5 however this is less variable and therefore not thought to be as immunologically important. High sequence homology is observed throughout the rest of the protein.

PorB makes up the majority of channels in the Neisserial outer membrane[356]. It is between 37 – 42 KDa and has a similar transmembrane structure to PorA. The surface exposed loops showing

the greatest variability are also the longest (loops 1, 5, 6, and 7)[318,313]. These loops contribute to the discontinuous serotype epitopes that are recognised by MAbs used for serotyping[85]. However, antibody-based typing is decreasingly used for meningococcal typing having largely been superseded by nucleotide sequence based typing[2,3,296]. PorB is an important component of OMV vaccines, including Bexsero and antibody responses against epitopes of PorB have previously been reported in up to 74 % of individuals following vaccination[73]. However, no response against PorB antigens was observed following immunisation with four doses of the Norwegian OMV vaccine[214]. More recently, the use of PorB as vaccine adjuvant to boost the immune response with fewer doses of the vaccine has been described[234].

The iron repressible FetA OMP is a member of the TonB dependant class of outer membrane proteins[60]. FetA, like PorA and PorB is also highly variable, and consists of a transmembrane β barrel structure with 13 surface exposed loops, of which loop 7 is the longest and most immunodominant variable region[322]. There are nine main families of FetA, consisting of over 400 variants, and more being identified regularly (http://pubmlst.org/*Neisseria*/FetA/). As for PorA and PorB, the high degree of variability observed with this protein is a sign that the FetA antigen is a target for a potentially protective immune response in humans and suggests it may be an important component of strain specific OMV vaccines[296,79]. However the diversity of FetA types presents several challenges in the design of a vaccine, with difficulties associated with achieving coverage over a wide range of strains[297,330]. Conversely, meningococci collections have been found to be dominated by a relatively small number of discrete combinations of PorA, FetA variants and clonal complexes. These antigenic types are found to persist of periods of time sufficient for the administration of vaccination programs and proliferate globally, suggesting the promise of FetA as a vaccines candidate[340].

The complement system is an important component of innate immune defences against the meningococcus, and deficiencies in the components of the alternative complement pathway lead to an increased risk of meningococcal disease[174]. The alternative pathways includes a mechanism which amplifies the deposition of C3b on the microbial surfaces using a positive

feedback loop, in addition to maximising the killing activity elicited by select anti-meningococcal Abs[15]. Factor H limits the unwanted activation of alternative pathway through irreversible disassociation of the alternative pathway C3 convertase, limiting C3 activation[174]. Meningococci fHbp is critical for meningococcal survival in the human host as it is thought to interact specifically with human factor H resulting in down-regulation of the alternative complement pathway through the down-regulation of deposition and inactivation of C3b on the meningococcal surface leading to serum resistance of the bacteria and protection form immune attack [272,125,146]. The fHbp protein has proved to be a virulence factor for N. meningitidis and a target for functional bactericidal antibodies[200]. Diverse activities have been reported for anti-fHbp antibodies, from inhibition of factor H binding, to increased meningococcal killing in strains with low level expression of fHbp. This protein is an important component of a meningitis vaccine that has undergone clinically trials and has been licensed for use as part of a multicomponent vaccine. However, some meningococcal isolates, collected from patients of invasive disease have been found to lack expression of fHbp, suggesting some meningococcal strains possess novel means of inhibiting complement activation in the absence of the fHbp[180]. As with the other proteins mentioned, multiple variants of this antigen have been described (http://pubmlst.org/Neisseria/fHBP/) [195,211].

3.2 Aims of this Chapter

The aims of this chapter were to:

- To develop cloning strategies to express these antigens as polyhistidine tagged proteins in *E. coli*;
- To purify the predominant variants of these antigens from hypervirulent isolates and commonly circulating meningococci as a basis for the development of a multiplex antibody binding assay.

3.3 Results

3.3.1 Amplification and Cloning of *porA*, *porB* and *fetA genes*

Sixteen *N meningitidis* isolates (Table 3.1) were cultured and gDNA was extracted prior to PCR amplification targeting either the *porA*, *porB*, *fetA* or *fHbp* gene as shown in Table 3.1. Meningococcal isolates encoding the required serotype, serosubtype or FetA type were identified in the NIBSC database. Isolates encoding two of more of these genes were picked where possible in the first instance and used for the extraction of gDNA. Isolates used for the amplification of *fHbp* were provided by the UK-MHPHLS for the purpose of this study.

A single PCR product corresponding to the predicted size of amplified bacterial DNA was observed for all genes amplified (Figure 3.2, Figure 3.3 and Figure 3.4). Figure 3.2 shows amplification of the *porB* gene encoding the 2a serotype with a PCR product of ~ 1 Kb. Figure 3.3 shows amplification of the *fetA* gene encoding the F3-6 variant with a PCR product of ~ 2.1 Kb, and the *porA* gene encoding the P1.19-1,15-11 serosubtype with a PCR product of 1.3 Kb. Figure 3.4 shows amplification of the *fHbp* gene encoding the v2.4 variant with a PCR product of ~ 800 bp. For most variants, 100 % of the colonies picked were successfully transformed. Successful transformation was found to be dependent on the quality of DNA resulting from amplification. For example transformation of the *porA* gene encoding the P1.5,2 variant of PorA and the gene encoding the 2b serotype of PorB was less successful due to failure of the PCR to amplify DNA encoding a single gene of interest. For isolates in which multiple PCR products were observed, a number of methods were utilised to optimise PCR amplification. These included increased stringency, decreased template DNA concentration, nested PCR or excision of the required band and subsequent gel purification. For PCR reactions where no band was produced, an alternate *Neisseria* (meningococcal) isolate was used in the first instance, followed by lower stringency conditions, including higher concentrations of magnesium chloride and a lower annealing temperature. For both of these variants, primers specific to the sequence of the isolate were ordered and used for PCR amplification. Eight serosubtypes of PorA were amplified in this way

along with three serotypes of PorB, five variants of FetA and four variants of fHbp. It was not possible to amplify a single PCR product for the 2b serotype of *porB* and *fHbp* variant 1.11. Possible explanations for difficulties in the amplification of these genes may include differences in secondary structure of DNA. The primer sequence used for the amplification of *porB* was based on the FAM18 sequence, believed to be the PorB 2a protein and may result in insufficient primer binding for successful amplification of the *porB 2b* gene. Poor quality of gDNA used for amplification or size of the insert may also explain difficulties in amplification of the required target however problems with amplification of these variants has not previously been reported, and the use of alternative gDNA and strain specific primers did not aid in cloning attempts.

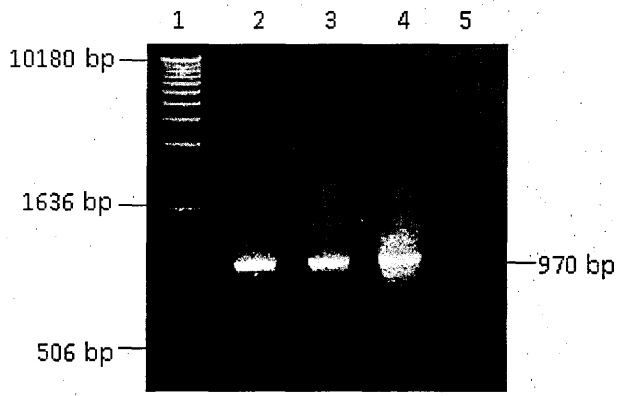


Figure 3.2 Agarose gel electrophoresis of PCR products following amplification of the *porB* gene using the PCR conditions as described in Section 2.3.3. Lane 1 shows the 1 Kb ladder (Invitrogen), Lanes 2 – 4 show PCR products resulting from amplification of *porB* from Nm 2918 (PorB 2a), with increasing concentrations of DNA used as a template, and Lane 5 contains a negative control.

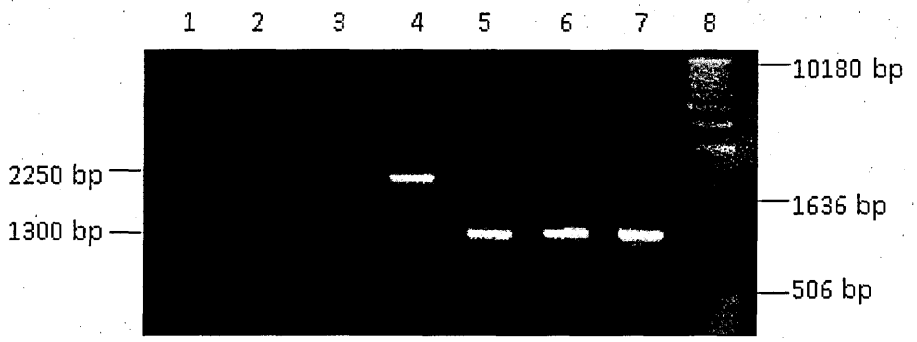


Figure 3.3 Agarose gel electrophoresis of PCR products following amplification of the *fetA* and *porA* genes, using the PCR conditions as described in Section 2.3.3. Lane 1 contains a negative control, Lanes 2 – 4 show PCR products resulting from amplification of *fetA* from Nm 2828 (F3-6) and Lanes 5 - 7 show PCR products resulting from amplification of *porA* from Nm 3069 (P1.19-1,15-11). Lane 8 contains a 1 Kb Ladder (Invitrogen) used to estimate the size of the PCR products.

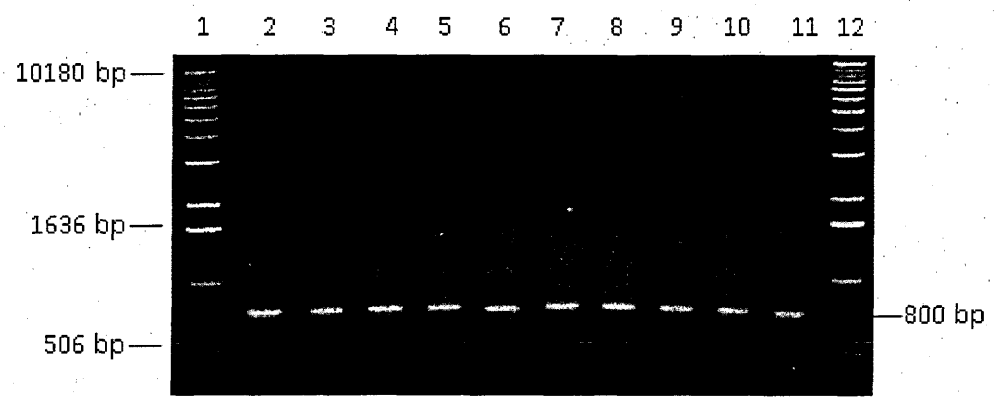


Figure 3.4 Agarose gel electrophoresis of PCR products following amplification of the *fHbp* gene using the conditions described in section 2.3.3. Lane 1 and 12 shows the 1 Kb ladder (Invitrogen), Lanes 2 – 11 show PCR products resulting from amplification of *fHbp* from Nm 3059 (v2.4) with extension temperatures increasing from 60 ˚C to 70 ˚C. All temperatures were found to produce a single PCR product of approximately 800 bp.

Table 3.1 Meningococcal strains used for extraction of DNA / gene cloning. Variants cloned have been shown in bold, whilst variants that could not be cloned are marked with a *. NT denotes where typing information was not available.

NIBSC Strain Number	Other Strain Number	Country of Origin	Serogroup	Serotype	Serosubtype	FetA type	fHbp type
2971	252433	UK	C	NT	**P1.19,15**	NT	NT
3069	M00-242911	UK-MPHLS[6]	B	NT	**P1.19-1,15-11**	NT	NT
2975	252437	UK-Sheffield	B	**2b***	**P1.5,2**	NT	NT
3080	M02-240879	UK-MPHLS	B	NT	**P1._,_**	NT	NT
2918	252493	UK-Chester	C	**2a**	P1.5-2,10-3	NT	NT
3061	M01-240149	UK-MPHLS	B	4	P1.7-2,4	NT	**1.4**
3059	M01-240013	UK-MPHLS	B	NT	P1.22,9	NT	**2.4**
3060	M01-240101	UK-MPHLS	B	NT	P1.19-1,15-11	NT	**1.11***
2851	H44/76	Norway	B	**15**	**P1.7,16**	**F3-3**	NT
2757	Z6426	New Zealand	B	**4**	**P1.7-2,4**	**F1-5**	NT
2828	Z1092	West Germany	A	NT	**P.1.5-2,10**	**F3-6**	NT
2794	Z4693	Norway	B	NT	**P1.5-1,2-2**	F1-7	NT
2758	Z6416/AK22	Greece	B	NT	NT	**F3-9**	NT

[6] Manchester Public Health Laboratory Service

NIBSC Strain Number	**Other Strain Number**	**Country of Origin**	**Serogroup**	**Serotype**	**Serosubtype**	**FetA type**	**fHbp type**
2769	Z4756	India	A	4,21	P1.5-2,10	**F5-1**	NT
3249	M05 240018	UK-MPHLS	B	NT	NT	NT	**1.9-3**
3248	M08 240016	UK-MPHLS	B	NT	NT	NT	**3.3**

3.3.2 Screening of transformed colonies

3.3.2.1 Colony PCR

Twelve single colonies were picked from each transformation and screened using colony PCR, with primers directed towards the T7 promoter and the T7 terminator sites, found on either side of the inserted gene sequence within the cloning region of the vector. Incorporation of the correct DNA fragment and vector was confirmed by a shift in the size of the PCR product from vector alone (360 bp) to the expected size of the vector with the complete DNA fragment, approximately 1.4 Kb for variants of the *porA* and *porB* genes, approximately 2.5 Kb for variants of the *fetA* gene (Figure 3.5) and approximately 1.1 Kb for variants of the *fHbp* gene. PCR products of the expected size were produced for all genes and variants. For the *porA* gene, 100 % of the transformed colonies picked were found to have successfully incorporated the gene insert. For *porB* and *fetA* approximately 60 % of picked colonies were found to have been successfully transformed. This may be due to the size of the FetA insert, which is considerably larger than the other target genes. Incomplete corporation of the *porB* gene may be a result of issues with amplification of the gene, with multiple bands observed in the gel following amplification of the target gene (Figure 3.2), it is possible that a smaller fragment had been inserted into the vector in preference to the larger *porB* fragment.

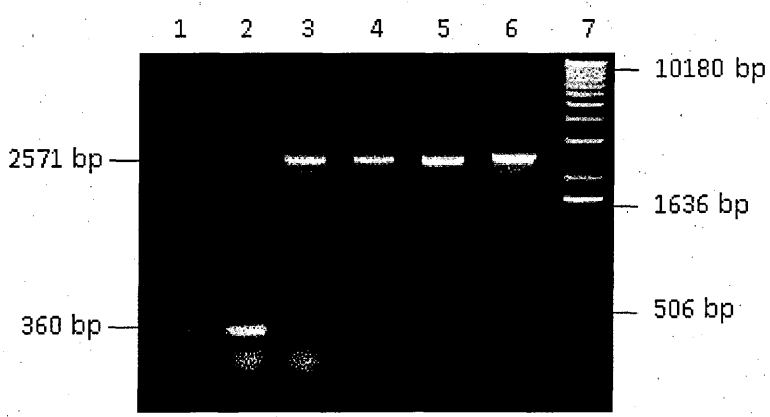


Figure 3.5 Agarose gel showing a shift in fragment size from vector alone (lanes 1 and 2) to colonies transformed with the *fetA* gene (lanes 3 – 6). Lane 7 contains the 1 Kb molecular weight ladder.

3.3.2.2 Sequencing

Plasmid DNA was sequenced using both forward and reverse primers and used to deduce the amino acid sequence. DNA and amino acid sequences were compared by alignment with known reference sequences for each antigen, and the variant was identified by comparison to the sequence of variants documented at the nesseria.org website (www.*Neisseria*.org). Four transformants were sequenced for each antigen variant to ensure that the gene insert was present in its complete form with no shifts in the reading frame or base changes as a result of the transformation process. Over 90 % of the *porA* transformants, 90 % of the *porB* transformants, 70 % of the *fetA* transformants and 100 % of the *fHbp* transformants sequenced were found to have successfully incorporated the correct DNA sequence. The majority of FetA transformants that were not sequenced successfully were as a result of an incomplete read as opposed to an incorrect read. Transformants with an incorrect sequence due to base insertions or deletions or lack of an N-terminal His-tag were discarded. The incorporation of the N-terminal His-tag into the correct reading frame, to allow for the expression of the full target protein was confirmed using the deduced amino acid sequence. Alignments for the deduced amino acid sequence for each antigen are given in the Appendix.

3.3.3 Protein Expression

A single clone was chosen for the expression of protein for each variant of PorA, PorB and FetA. An initial 100 ml culture was inoculated for the expression of PorA, PorB and FetA proteins in the first instance. Bacterial growth was estimated by measurement of OD600 using a cell density meter over a five hour period following induction with IPTG. The growth curve was plotted (Figure 3.) and highlighted the effect of induction of transcription on cell growth. The maximum cell density for cultures induced with a final concentration of 1 mM IPTG, was achieved two hours following protein induction.

Samples of cells were collected hourly and analysed using SDS-PAGE to check protein expression, as shown in Figure 3. Expression of the target protein was seen as early as 1 hour following IPTG

induction, and was absent within the control samples. A lower level of contaminating proteins was observed in the IPTG induced culture than in the control culture due to the induced expression of the target protein. Similar results were observed for each protein purified in this way. The level of protein expression appears to be constant at all time points through the induction period, however the loading volume was standardised according to the OD600 measurement. Further large scale bacterial cultures (500 ml) were used for purification of the remaining antigenic variants and were induced with IPTG for the optimal induction time of 2-3 hours.

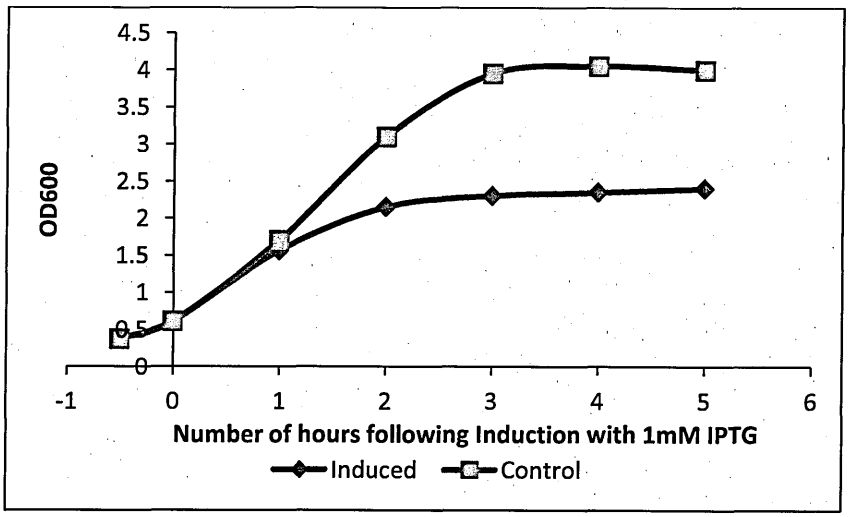


Figure 3.6 Growth curve showing the effect of Induction with 1mM IPTG on a culture of PorA variant P1.7,16 (Nm 2851). A single culture was grown to an OD600 of 0.6 and was then split into 2 cultures, one of which was induced with IPTG and the other used as a control.

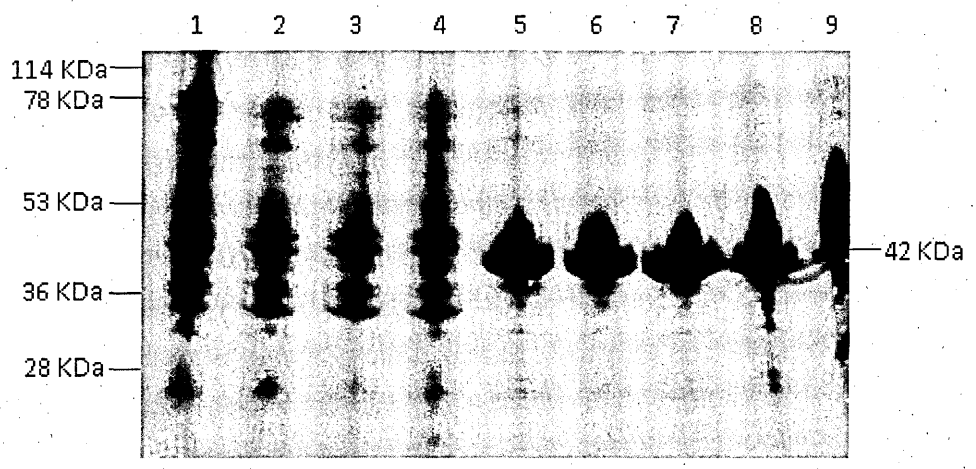


Figure 3.7 An SDS gel showing the effect of Induction with 1 mM IPTG on a culture of PorB variant 15 (Nm 2851). Lanes 1 – 4 show the WCL of the control culture taken at 2 – 5 hour time points, whilst lanes 5 – 9 show the WCL of the induced cultures taken at 1 – 5 hour time points following the addition of IPTG.

3.3.4 Protein Purification

The wet weight of the pellet was measured at several stages during the protein purification process. . This was achieved by using a pre-weighed centrifuge tube to weigh the pellet after removal of the supernatant following centrifugation. As some of the supernatant is likely to still be contained with the centrifuge tube, this is termed the wet weight of the pellet. The cell pellet wet weight was measured following harvest of whole cells following IPTG induction, and will include the weight of the *E.*coli cell wall and outer membrane components. The wet weight of the inclusion bodies was measured following purification of the inclusion bodies from cell lysates using the Bugbuster Protein Extraction Reagent. The percentage of inclusion body wet weight purified from the cell pellet wet weight ranged from 13.3 % for the P1.7,16 serosubtype of PorA to 39.3 % for the PorB serotype 2a (Table 3.2).The amount of total protein purified from 500 ml of cell culture ranged from 36.73 mg for the F3-9 variant of FetA to 213.75 mg of the P._,_ variant of PorA following the solubilisation of the inclusion bodies. With a similar level of purified protein observed for the variants of each antigen. The percentage of total protein purified from the wet weight of the inclusion body ranged from 7.2 % for the PorB 2a serotype to 30.5 % for the P1.7,16

serosubtype of PorA. Again the weight of the inclusion body was measured as a wet weight, and may have accounted for these variations.

Following the final column purification step, the yield of pure protein ranges from 4.5 mg of FetA to 110 mg of the P1._,_ PorA variant. There was a significant difference in the percentage of final purified target protein in relation to the total inclusion body proteins, and ranged from 9 % for the F3-6 variant of FetA to 88.5 % for the P1.5-1,2-2 serosubtype of PorA. PorA proteins were seen to be purified in higher concentrations and as a percentage than the PorB proteins. The lowest amount of protein was purified for the FetA antigens. This may be due to several reasons. The large molecular weight of the FetA proteins in relation to the PorA and PorB may have resulted in a decrease in protein production. There are several stages between purification of total proteins from the inclusion bodies, and the final protein, including a dialysis step and column purification. Some of the FetA protein may not have been bound to the His-Bind column sufficiently prior to washing and have washed through the column prior to elution. The FetA proteins, in particular, were found to have issues with precipitation occurring during dialysis. Whilst the concentration of FetA proteins found in the resolubilised precipitate was low, this may account for the reduced yield in protein observed with this antigen.

Table 3.2 Total protein purified from 500 ml of cell culture induced with 1mM IPTG following His-Bind column purification. Inclusion bodies are expressed as a percentage of the cell wet weight. The total protein purified following resolubilisation of the inclusion bodies is expressed as a percentage of weight of the inclusion body. Estimated purified protein is expressed as a percentage of the total inclusion body proteins.

Antigen	Variant	Cell wet weight	Inclusion Bodies		Total protein		Est. Purified Protein	
		(mg)	(mg)	(%)	(mg)	(%)	(mg)	(%)
PorA	P1.19,15	2550	420	16.5	91.77	21.9	50.5	55.0
	P1.19-1,15-11	2580	420	16.3	64.47	15.4	39	60.5
	P1.5,2	2050	670	32.7	106.52	15.9	65	61.0
	P1.5-1,2-2	2260	430	19.0	61.04	14.2	54	88.5
	P1.5-2,10	2330	470	20.2	100.11	21.3	63.5	63.4
	P1.7,16	2550	340	13.3	103.55	30.5	48	46.4
	P1.7-2,4	2120	640	30.2	101.95	15.9	30	29.4
	P1._,_	4250	1000	23.5	213.75	21.4	110	51.5
PorB	PorB 2a	1680	660	39.3	50.37	7.6	29.5	58.6
	PorB 4	2170	510	23.5	83.18	16.3	39	46.9
	PorB 15	2900	390	13.4	66	16.9	44	66.7
FetA	F1-5	2150	640	29.8	89.71	14.0	13	14.5
	F3-3	1410	300	21.3	49.64	16.6	8	16.1

Antigen	Variant	Cell wet weight	Inclusion Bodies		Total protein		Est. Purified Protein	
		(mg)	(mg)	(%)	(mg)	(%)	(mg)	(%)
FetA	F3-6	1710	400	23.4	66.49	16.6	6	9.0
	F3-9	1860	270	14.5	36.73	13.6	4.5	12.3
	F5-1	1910	460	24.1	96.06	20.9	9	9.4

3.3.4.1 SDS Gel Electrophoresis/Purity of Protein

Samples collected from the each stage of the purification process were analysed using SDS-PAGE to assess purity of the protein and the efficiency of the purification process, in order to optimise the purification method (Figure 3.).

As previously observed, IPTG induces expression of the target protein with increased expression levels observed at successive sampling points (lanes 1-3, Figure 3.). In addition to the target protein, low levels of many other bacterial proteins were also observed, however most of these contaminants were removed following inclusion body purification. The localisation of the target protein within the inclusion bodies was confirmed in lane 4 and 5 (Figure 3.) where no protein bands are observed in the supernatant following removal of the inclusion bodies. Protein was visible (lane 6, Figure 3.) following resolubilisation of the inclusion bodies, whilst many of the other bacterial proteins were not. This confirmed the high expression of the recombinant protein in the absence of the signal peptide and the removal of contaminating bacterial proteins from the proteins extract.

Once the protein extract was loaded on the His Bind resin column very little protein was lost, although a small amount was visible in the wash fraction (lane 10, Figure 3.). This may have been a result of overloading of the column, or due to the increased concentration of imidazole present in the wash buffer, and elution of any loosely bound proteins. Elution with 1 M imidazole produced a single band of the expected size for each of the purified proteins. High level of protein purity was observed as there were no contaminating bands observed on the SDS PAGE gel following analysis. A similar result was obtained for the purification of all PorA, PorB and FetA variant proteins using this method. A difference in the size of the protein band was observed before and after the final dialysis step. This may have been due to insufficient boiling of the protein extract prior to loading on the gel or as a result of differences in the composition of Elution buffer and Dialysis buffer 2.

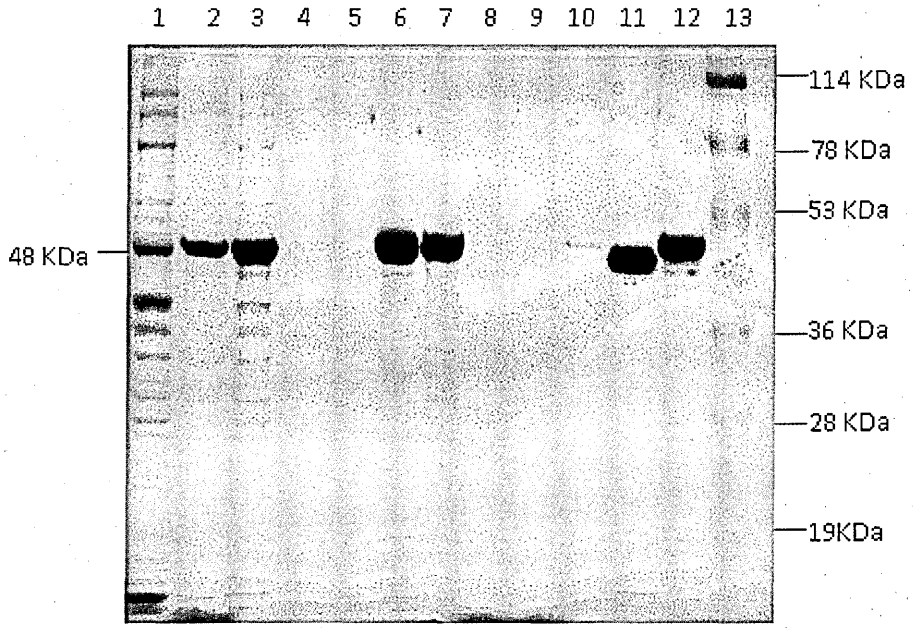


Figure 3.8 An SDS PAGE gel image showing the purification of P1.5-1,2-2 (Nm 2794) at various stages of the purification process. Lane 1, 2 and 3 show the WCL at 0, 1 and 2 hours following the addition of IPTG, respectively. Lane 4 shows the resulting supernatant following removal of the inclusion bodies with the supernatant from a subsequent wash step shown in lane 5. Lane 6 shows the purified protein extract prior to dialysis in Dialysis Buffer 1. Lane 7 shows the extract following dialysis in Dialysis Buffer 1. Lanes 8 to 12 shows flow through from various stages of the His Bind column purification. Lane 8 shows the flow through following application of the protein extract to the column. Lane 9 shows the flow through following application of the binding buffer to the column. Lane 10 shows the flow though after the column is washed. Lane 11 shows the eluted purified protein prior to dialysis in Dialysis Buffer 2 and lane 12 shows the final purified protein extract following dialysis in Dialysis buffer 2. Lane 13 is the SDS-PAGE Standards, Broad range marker (Bio-Rad).

3.4 Discussion

The objective of the part of the project described in this chapter was to clone, express and purify His-tagged proteins from variant strains of the major meningococcal outer membrane proteins, PorA, PorB and FetA. These purified antigens are to be used to produce a panel of protein coated microspheres to be used in the development of multiplex antibody binding assays.

This objective was achieved with the successful cloning, expression and purification of all target antigens including eight serosubtype strains of *porA*, three serotype strains of *porB* and five

variant strains of *fetA*. Although the fHbp antigen was not originally considered a target antigen for the purpose of this study, four variant strains of *fHbp* were also cloned. These strains were not available until later in the project and were not used to express protein due to time constraints. Two antigenic variant strains could not be cloned. The strain of variant encoding for the PorB serotype 2b protein could not be cloned following PCR optimisation. This may have been due to differences in the secondary structure of DNA, however even with the use of DNA from alternative isolates and the use of strain specific primers for the PorB type 2b protein, cloning was unsuccessful. Alternative isolates were not available for the fHbp variant 1.11 so this variant strain was discarded.

A method that has largely been adopted for the purification of recombinant proteins includes the expression of protein as inclusion bodies, resolubilisation and refolding using detergents[264]. A number of Neisserial OMPs have been purified using this method including PorA, PorB and FetA[206,143,161]. The most widely used method for purification of recombinant proteins utilises the His-tag and allows for purification of the tagged protein using IMAC[179] with an increasing concentration of imidazole to allow for any loosely bound proteins to be washed away before elution of the purified protein. The use of commercially available cloning and purification kits ensures optimal and consistent overexpression and purification of recombinant proteins. Cloning and transformation of antigenic variant strains was performed using the pET expression system. Use of the pET vector system for the overexpression of His-tagged OMPs such as PorA has previously been described[129] with protein purity and yields comparable to those described in this thesis. FetA proteins were found to have a specific issue with this method with precipitation observed for several of these proteins during the removal of urea through dialysis. Protein precipitation during dialysis has previously reported[129] however, despite the low amount of protein purified for these antigens, precipitation was found to be due to urea coming out of solution as opposed to protein precipitation. Analysis of both the supernatant and the resolubilised precipitate, using a BCA protein determination assay, confirmed that whilst protein was found in the supernatant, no protein was present in the precipitate. No precipitation was

observed following dialysis for any of the PorA or the PorB proteins and all purified proteins were found to be of the expected size, and purity. The lowest concentration of protein was purified for the FetA proteins in comparison to the PorA and PorB proteins and is likely to be a result of differences in the size of the proteins. FetA is a larger protein than both PorA and PorB and would have been more difficult to express.

The use of the Triton X-100 detergent in the final elution buffer to stabilise the recombinant membrane proteins[181] hampered refolding studies and although dialysis was performed following elution, complete removal of Triton from the purified protein extract was not possible. These minimal amounts of Triton X-100 were found to interfere with many of the assays used to confirm correct refolding of the target proteins. Whilst methods such as Circular Dichroism Spectrophotometry under near and far UV[181] were attempted (with Dr. Angela Martino), these could not be used to confirm the protein had been refolded correctly. However, the proteins have been used for separate studies in the laboratory and refolding of the protein for the correct exposure of immunogenic epitopes has been confirmed. Immunisation studies (performed by Dr. Hannah Chan as part of a different project) in mice have shown that purified PorA and FetA proteins are in the correct conformation as to elicit the production of bactericidal antibodies (data not available), and are thereby sufficiently refolded for use in the development of the multiplex assay. Further proof of the confirmation of the PorA binding epitopes, using monoclonal antibodies are shown in Chapter 4. Purified recombinant PorB proteins have been shown to bind to MAbs, confirming the correct refolding of these antigens using slot blot assays. The FetA proteins have also been used to elucidate the structure and function of FetA[265,266]. In addition to the studies investigating the structure and function of OMPs, the antigens purified in this chapter potentially have a variety of uses. These include use of the antigens as standards reagents for use in immunogenicity assays including the SBA assay and ELISA; meningococcal typing schemes; and in immunisation experiments for the production of specific monoclonal or polyclonal antibodies. These antigens can also be used in the development of new vaccines as vaccine candidates, or for the detection of specific immune responses following vaccination.

For the purposes of this thesis, these antigens were found to be pure and of sufficient concentration to be carried forward for conjugation to microspheres in order to produce a panel of antigen labelled microspheres for use in the development of a multiplex Liquichip assay to measure serological responses to specific meningococcal antigens.

Chapter 4. Development and Validation of a multiplex assay for the detection of serological responses to PorA

4.1 Introduction

The current method used for the quantification of PorA serum antibodies relies on their reactivity with the epitopes of the protein in ELISA or dot-blot based methods[13,260,344]. The ELISA is a specific, and reproducible assay and provides a good assessment of immune responses but is only an indirect measure of protective immunity with no information about antibody functional activity[281]. The ELISA, whilst suitable for the measurement of multiple samples for a single antigen, cannot be used for the quantification of multiple antigens within a single serum sample. The need for multiple ELISAs in addition to the multiple serum dilutions required for each antigen mean that the ELISA becomes time consuming and requires relatively large volumes of both the target antigen for plate coating and sera[332,167].

Multiplex flow cytometric immunoassays have been developed which allow for the measurement of multiple antigens within a single serum sample[41,332]. This method has all the advantages of the ELISA for the measurement of serological responses with additional benefits of reducing the volume of sera and the amount of time required for the analysis of multiple antigens and increasing sample throughput[41,332,167]. A method that allows for the addition of new variants as required in a simple and effective manner would also be beneficial. Use of a multiplex format assay of this type could be used for measuring antibody responses in the evaluation of new vaccines, such as OMV, combination, and protein based vaccines. Serological responses to meningococcal and pneumococcal capsular polysaccharides have been successfully analysed using this technology[167,168,166,275].

A multiplex system, such as the LiquiChip assay can be used to simultaneously quantify multiple antigens within a single assay[103]. This method uses microspheres in place of the solid support used for ELISA. Each microsphere set is labelled with a combination of red/infrared dyes, resulting in a unique fluorescent signature. Capture molecules can be coupled to different

microsphere sets and mixed together enabling the individual detection of multiple antigens simultaneously[41]. A secondary green reporter molecule, phycoerythrin, is conjugated to an antibody specific for the antibody of interest and is used to quantify the reaction. The process is summarised in Figure 4.1. Data is collected using a LiquiChip workstation, a flow cytometer designed for this purpose, which quantifies the fluorescent signals from each microsphere. The red/infrared fluorescence is used to differentiate between microsphere sets, whilst the green fluorescence is used to quantify antibody binding.

Due to the nature of the microsphere and the wide scope of the Luminex technology, there is the potential for a range of capture molecules to be used for labelling of the microspheres including cytokines, proteins, polysaccharides, lipopolysaccharides, antibodies and nucleic acids. There are several methods by which the protein may be attached to the microspheres. Direct conjugation may be performed using carboxylated or activated microspheres, through the amine groups of lysine residue, or the thiol groups of cysteine residues within the protein. Direct conjugation was not used as this method may reduce immunogenicity of the primary antigen and limit signal amplification. Indirect methods involving coupling of a capture molecule, such as nickel-nitriloacetic acid (Ni-NTA) or Penta-His antibodies, to bind N' terminal tagged proteins. These methods allow for correct exposure of the epitope for antibody binding, resulting in increased sensitivity and signal amplification. Avidin coating can also be used to bind biotinylated proteins using indirect conjugation. Use of Ni-NTA as a capture molecule for the affinity purification of 6x his-tagged proteins[56] has become a universal method for the purification of recombinant proteins, with binding between the nickel ion and the 6x his-tag shown to have high affinity and selectivity[271,360]. The Ni-NTA/His-tag is not dependent on three-dimensional confirmation and can be used under native and denaturing conditions. This is a valuable and flexible metal chelation system and has been exploited for a number of purposes with Ni-NTA /His-tag binding used for a range of methods including: protein- labelling schemes[360]; immobilisation of 6x his-tagged proteins on agarose beads[271]; microtitre plates[271] and microsphere beads[170] such as those used in this study. Ni-NTA microspheres have been developed by LiquiChip for the universal

directed immobilisation of 6x His-tagged proteins using the Luminex assay, and for the purpose of this study, Ni-NTA labelled microspheres were conjugated to the His-tag of the purified PorA proteins.

4.2 Aims of this chapter

The aims of this chapter were:

- To develop a multiplex assay, based on Luminex technology using microspheres labelled with purified his-tagged PorA proteins to be used for the detection of IgG responses to specific serosubtypes of PorA simultaneously;
- To optimise this assay for the measurement of antibody responses using mouse MAbs and human sera;
- To demonstrate the validity of this assay, including specificity, reproducibility, and sensitivity of the assay.

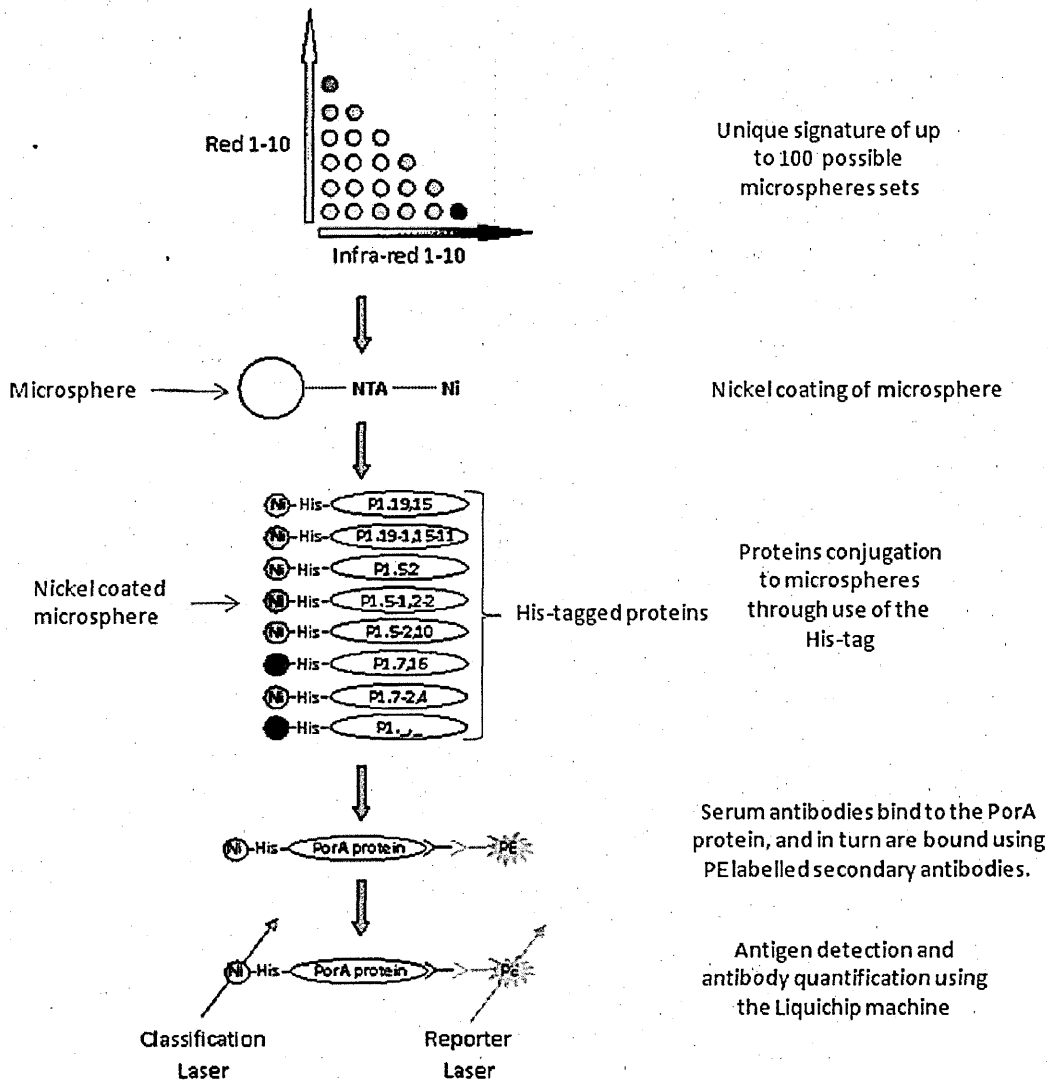


Figure 4.1 Summary of the LiquiChip assay. Microspheres are selected from a range of 100 uniquely labelled microspheres and coated with NTA moieties, which in turn are bound to nickel ions. The nickel of these metal chelator complexes bind to the His-tag, expressed as part of the PorA proteins resulting in a protein coated platform. Protein labelled microspheres are used to bind antibodies in serum. Phycoerythrin labelled secondary antibodies, specific to serum antibodies, is added and the microspheres are analysed using the LiquiChip workstation. A red classification laser detects the microsphere set used, whilst a green reporter laser quantifies the reaction.

4.3 Source of sera used

Sera used in this chapter were obtained from several sources as listed in Table 4.1. Serum from the VEU was collected as part of an NVEC study, from healthy laboratory workers vaccinated with three doses of either the P1.7,16 OMV vaccine (NW) or the P1.7-2,4 OMV vaccine (NZ). Serum

from this collection was selected for use in this study based on the assumption of high titres of anti-PorA antibodies, present in serum following vaccination with one of two PorA vaccines. Serum 03/118 was an unvaccinated serum sample from an individual healthy laboratory worker, known to contain high titres of anti-PorA antibodies directed against multiple PorA serosubtypes. Selection of these sera were also based on locality, with both these sets of sera maintained in the NIBSC collection and have previously been used in the laboratory.

Table 4.1 Source of sera used in this chapter.

Source	Serum	Sera Samples
VEU (Manchester)	P1.7,16 OMV vaccine (NW) P1.7-2,4 OMV vaccine (NZ)	17 samples 9 samples
NIBSC	03/118 Mouse Antibodies (detailed in Table 4.2)	1 sample 9 monoclonal

Table 4.2 Monoclonal antibodies used in this chapter.

Serosubtype	Monoclonal
P1.19	WRAIR 7A2-11
P1.5	NVI MN22A19.9
P1.7	NVI MN14C11.6
P1.15	NVI MN3C5C
P1.10	NVI MN20F4.17
P1.4	NVI MN20B9.34
P1.16	NVI MN5C11G
P1.2	NVI MN16C13F4
Universal	05/194

The Universal is an anti-meningococcal PorA monoclonal mouse antibody. The epitope for this antibody is undefined but has been found to cross-react in whole cell ELISA and co-agglutination.assays.

4.4 Results

4.4.1 Optimisation of the PorA LiquiChip assay

In order to determine the minimum number of bead required for the detection of anti-PorA antibodies in each serum sample, the Liquichip assay was performed using 5000, 2500 or 1250 microspheres for each PorA panel serosubtype, per assay point. Pooled human serum, previously collected from healthy laboratory workers following immunisation with three doses of the Norwegian (P1.7,16) OMV vaccine was used to produce standard curves, plotted using the mean fluorescence intensity (MFI) recorded for each microsphere subset (Figure 4.2). Similar MFI values were obtained at lower sera concentrations, regardless of the total number of microspheres used. As the concentration of sera increased, MFI values for both the 5000 and the 1250 microspheres per data point appeared to plateau, or in some cases, such as the P1.5-2,10 PorA serosubtypes, actually decreased. This was not observed when 2500 microspheres were used. The highest MFI, for the panel of PorA labelled microspheres, were recorded using 2500 microspheres per assay point. This number of microspheres therefore provided the widest dynamic range of concentrations at which the assay could be performed. Additionally, the minimum microsphere count of 100, per data point was not achieved for all assay points when 1250 microspheres were used (data not shown).

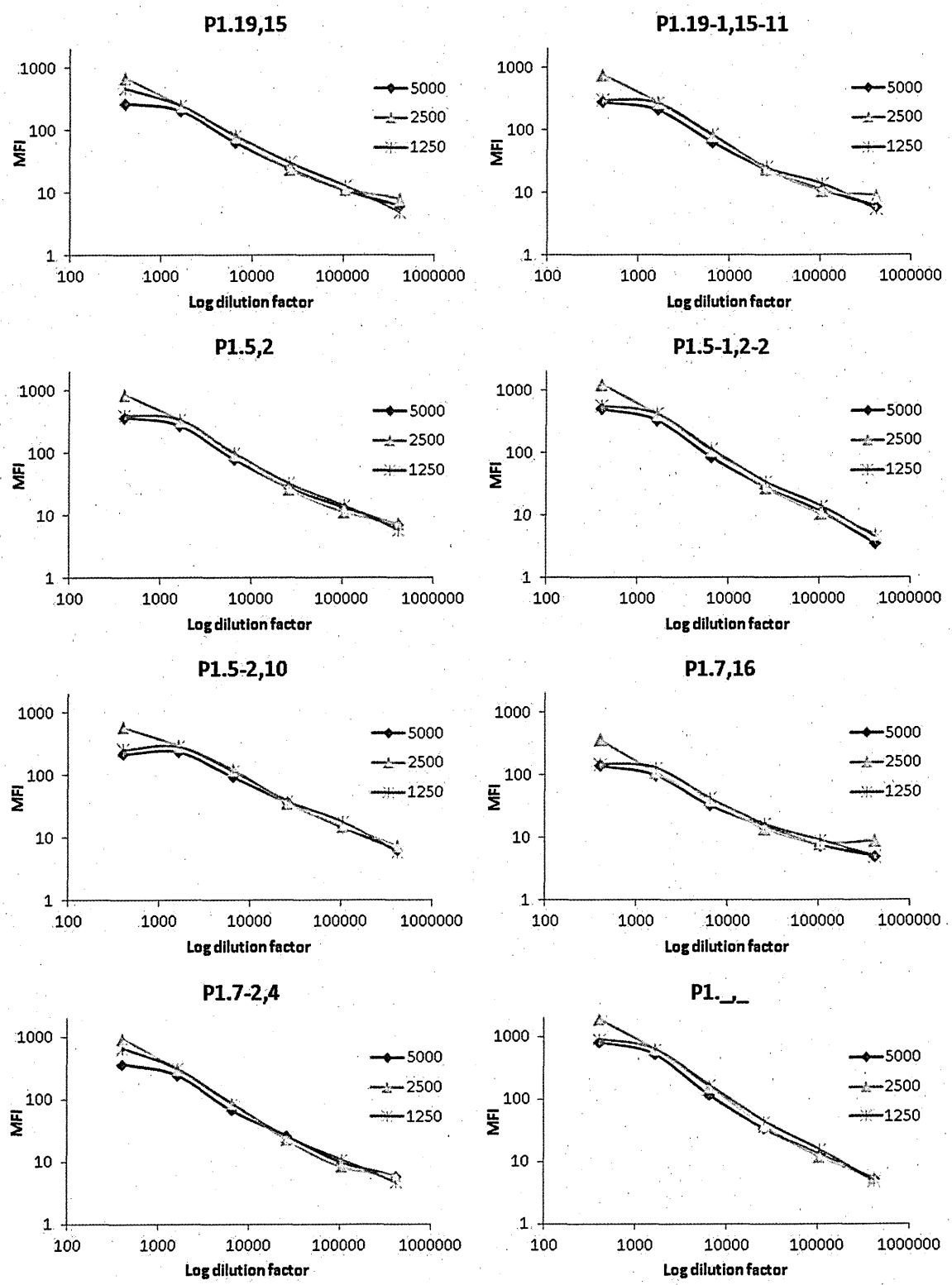


Figure 4.2 Standard curves for pooled serum from healthy laboratory workers vaccinated with the P1.7,16 OMV vaccine. The LiquiChip assay was performed on pooled human sera previously collected from healthy laboratory workers following immunisation with three doses of the P1.7,16 OMV vaccine. Two replicates of pooled sera sample were tested using 1250, 2500 or 5000 microspheres as two independent assays. MFI values were recorded and standard curves were plotted for the panel of PorA coated microspheres.

4.4.2 Assay Specificity

4.4.2.1 Specificity using mouse MAbs

Specificity of the multiplex assay was assessed using MAbs corresponding to VRs of the purified PorA serosubtypes proteins. These are the mouse monoclonal antibodies used for the PorA serosubtyping scheme. MAbs were found to react only with the microspheres coated with the corresponding PorA serosubtypes and closely related variants (Table 4.3). No reaction was observed between the MAbs and microspheres coated with PorA proteins from different serosubtype families.

The P1.19 MAb was found to cross-react with both P1.19 and the P1.19-1 serosubtypes. The P1.5 MAb was found to cross-react with both P1.5 and the P1.5-1 serosubtypes, however this antibody did not exhibit any response to the P1.5-2 serosubtype. The P1.7 MAb was found to cross-react with both P1.7 and the P1.7-2 serosubtypes. The P1.2 MAb was found to cross-react with both P1.2 and the P1.2-2 serosubtypes. Whilst the P1.15 MAb showed only a limited response with the P1.15-11 serosubtype. MAbs corresponding to P1.10, P1.16 and P1.4 were only found to react with the expected serosubtypes of PorA.

The Universal antibody, so named because of its ability to recognise multiple PorA epitopes, was found to respond to four of the eight the PorA labelled microspheres, including the P1.5,2; P1.5-1,2-2; P1.7-2,4 and the P1.7,16 labelled microspheres. Again, no cross reactivity was exhibited between the P1.5 and the P1.5-2 serosubtypes. The Universal antibody was found to cross react with the P1._,_ labelled microsphere, whilst no IgG response was observed between this protein and any of the other MAbs.

Table 4.3 Multiplex assay specificity. Reactivity of MAbs with different antigenic variants of PorA (recorded as MFI). Values highlighted in bold indicate a positive reaction. Three replicates of each MAb were used at a dilution of 1:1000 as a single experiment.

		Protein labelled microspheres							
		P1._,_ (52)	**P1.19,15 (36)**	**P1.19-1,15-11 (32)**	**P1.5,2 (58)**	**P1.5-1,2-2 (34)**	**P1.5-2,10 (50)**	**P1.7,16 (54)**	**P1.7-2,4 (38)**
MAb	**P1.19**	6	**4664**	**4483**	9	6	17	7	5
	P1.5	6	8	9	**2030**	**6105**	9	9	6
	P1.7	6	10	11	8	5	13	**1307**	**1003**
	P1.15	6	**5425**	55	9	5	14	6	7
	P1.10	7	8	9	8	7	**3411**	8	6
	P1.4	5	8	10	8	5	8	6	**1633**
	P1.16	8	21	26	12	9	31	**4329**	11
	P1.2	9	13	14	**7648**	**9745**	19	10	7
	Universal	**14120**	15	11	**10142**	**12567**	16	**2630**	**9526**

4.4.2.2 Inhibition of MAbs with purified proteins

A pool of MAbs was produced by mixing equal volumes of each MAb shown in Table 4.3, with the exception of the Universal antibody. This MAb mixture was pre-incubated together with a purified PorA protein, resulting in the specific inhibition of binding between the corresponding PorA labelled microsphere set and the MAb mixture (Figure 4.3).

Whilst pre-incubation with the P1.19,15 protein was shown to inhibit binding to both the P1.19,15 and P1.19-1,15-11 microsphere sets, pre-incubation with the P1.19-1,15-11 protein however showed limited inhibition with the P1.19,15 microsphere set and complete inhibition with the P1.19-1,15-11 PorA labelled microsphere set. Pre-incubation with the P1.5-2,10 protein resulted in limited inhibition of the IgG response to the P1.5-2,10 labelled microspheres, but did not exhibit any effect on either the P1.5,2 or the P1.5-1,2-2 labelled microspheres. Inhibition was not observed between the P1.7 and the P1.7-2 minor variants with the IgG response thought to be due to binding of antibodies corresponding to the VR2 P1.16 and P1.4 regions of PorA protein.

There are several possible explanations for the incomplete binding exhibited between several of the PorA antigens and the corresponding MAbs. These include a high concentration of MAbs in relation to the antigen; antibody affinity; and competition between binding sites on the free protein and the microsphere bound protein. MAbs are produced in mice in response to a human antigen, and this may affect the mechanism of action between the antibody and the inhibiting protein leading to incomplete inhibition.

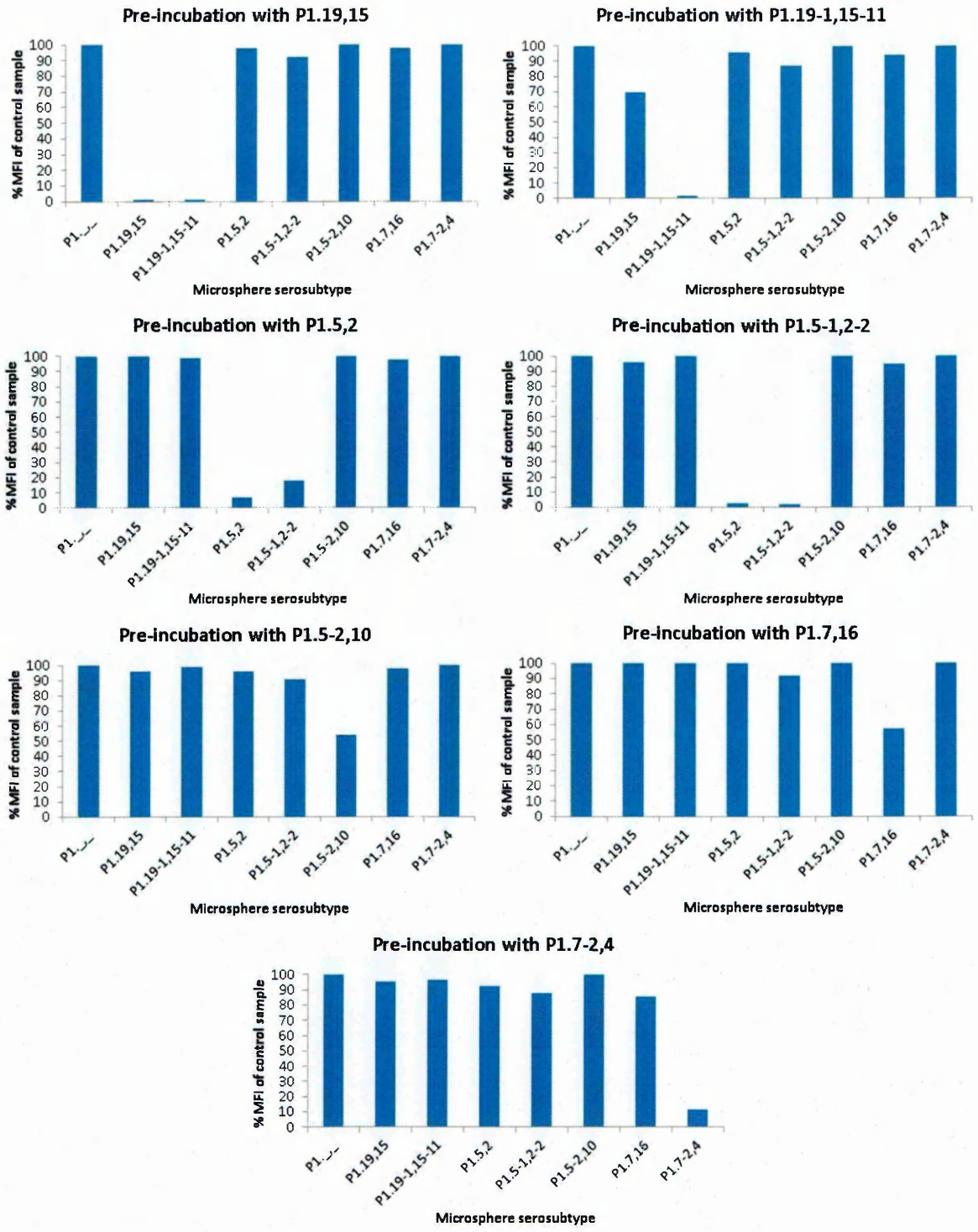


Figure 4.3 Inhibition of MAbs with purified proteins. A mixture of MAbs was pre-incubated, for 1 hour at room temperature, with purified 100 µg/ml PorA proteins corresponding to the microsphere sets used. Control sample of uninhibited MAbs were also tested using the LiquiChip assay, and the effect of inhibition is expressed as a percentage of the control sample. Results are expressed as the average of two replicate samples run on two independent assays.

4.4.2.3 Inhibition of human serum responses with purified proteins

Serum collected from a healthy laboratory worker (NIBSC 03/118) was incubated with purified PorA proteins, prior to running on the LiquiChip assay.

Inhibition with every protein resulted in a reduction in binding response to the corresponding microsphere set (Figure 4.4). IgG responses to the P1._,_ labelled microsphere were largely unaffected following incubation with all of the PorA proteins. The effect on other microsphere sets however varied. Inhibition with both P1.19,15 and P1.19-1,15-11 proteins resulted in a decrease in antibody binding for all microspheres to a similar extent, with the exception of the P1._,_ labelled microspheres. Some inhibition was observed in response to all microsphere sets following incubation with either the P1.5,2 or the P1.5-1,2-2 protein, however, the most significant decrease in IgG response in both cases was observed for binding to the microsphere sets labelled with the homologous proteins. The P1.5-2,10 protein exhibited a blocking response on the P1.5-1,2-2 and the P1.5,2 microsphere sets, which is thought mainly to be attributed to the VR1 P1.5. More specific inhibition was observed following incubation with the P1.7,16 and the P1.7-2,4 proteins with inhibition due to the VR1 P1.7, as demonstrated using MAbs.

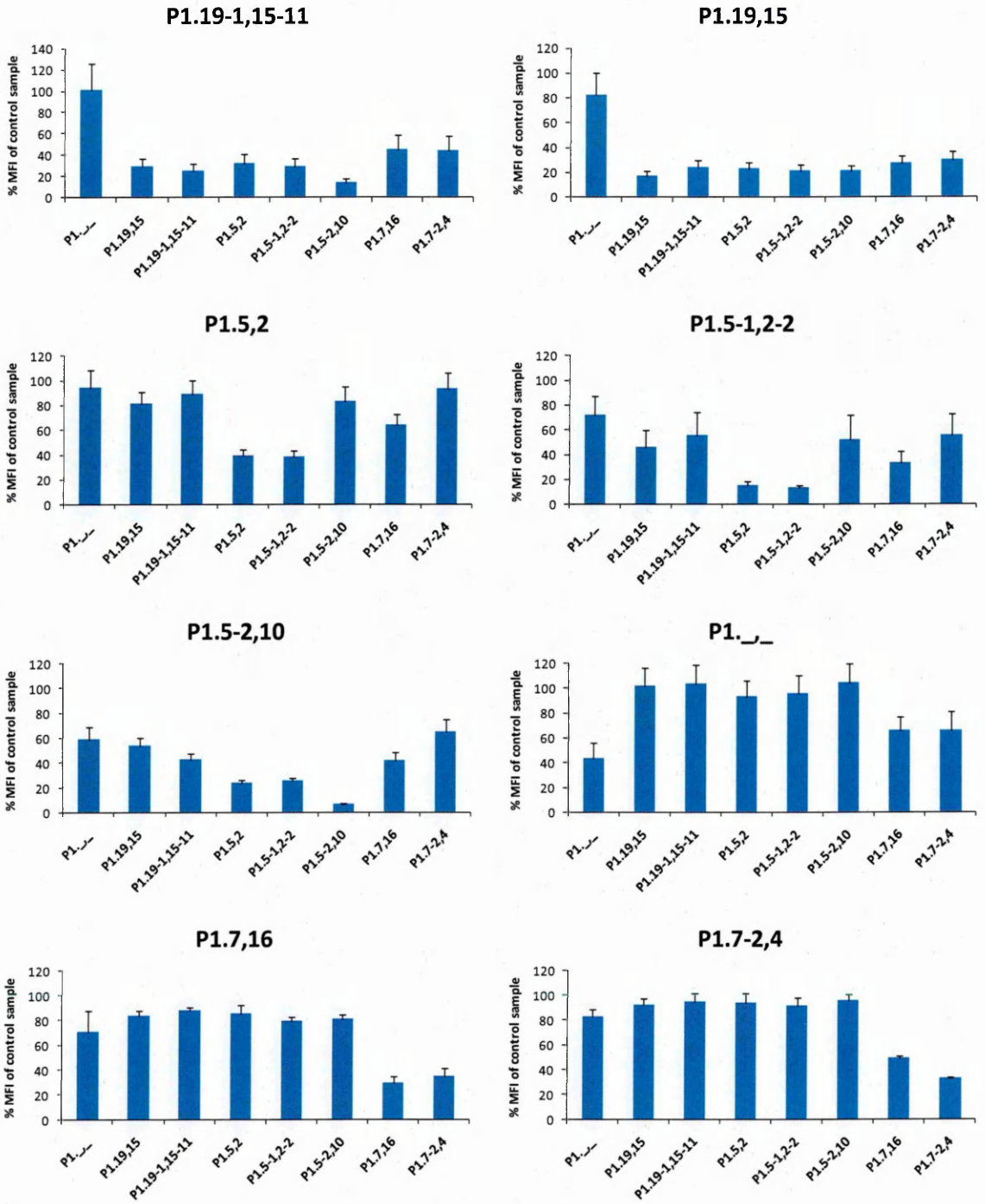


Figure 4.4 Inhibition of human serum with purified proteins (error bars represent 95 % confidence intervals). A human serum sample (03/118) was pre-incubated, for 1 hour at room temperature, with purified PorA proteins corresponding to microsphere sets used. An uninhibited sample of the 03/118 human serum was used as a control and also tested using the LiquiChip assay. The effect of inhibition is expressed as a percentage of the control sample. These results are expressed as the average of two replicate samples run on three independent assays.

4.4.3 Assay reproducibility

Sera from healthy laboratory workers immunised with three doses of either the P1.7,16 OMV or the P1.7-2,4 OMV vaccine (from the SEU, Manchester, Table 4.1) were pooled and used to produce a standard curve for the calculation of antibody concentrations in human sera using the liquichip assay. Six dilutions of the sera were used for the production of the standard curve and ranged from a 1:100 dilution of the pooled sera, to a 1:24300 dilution using a 1:3 dilution series. As the actual concentration of anti-PorA antibodies in the standard serum was not known, an arbitrary concentration was assigned for each of the serosubtype specific anti PorA antibodies with the highest concentration of standard used (1:100 dilution) assigned an arbitrary concentration of 10 ng/ml. The lowest concentration of standard (1:24300) was assigned an arbitrary concentration of 0.04 ng/ml. A standard curve was produced by plotting the MFI for each dilution of standard against the concentration of that standard. A line of best fit was applied to these values and allowed for an arbitrary sample concentration to be determined for all sera samples based on the MFI values measured using the Liquichip assay. A different standard curve was produced for each serosubtype specific anti-PorA antibody measured using this assay. Use of an arbitrary concentration allowed for the comparison of serosubtype specific anti-PorA titres in samples over several assays, however, the concentration of antibodies directed against different PorA serosubtypes cannot be compared.

Assay reproducibility was determined using replicate sera samples from a pool of healthy laboratory workers following immunisation with three doses of the human P1.7,16 OMV and a pool of healthy laboratory workers following immunisation with three doses of the human P1.7-2,4 OMV vaccine (from the SEU, Manchester, Table 4.1). The coefficient of variation (CV) for each of the panel of PorA labelled microspheres was determined for each set of repeat samples.

4.4.3.1 Intra-Assay variability

Intra- assay variability was determined using seven replicates, at three dilutions, of sera samples from a pool of healthy laboratory workers immunised with the P1.7,16 OMV vaccine. The assay

was found to have little intra-assay variability with the CV ranging from 3.82 % for the P1.19,15 microsphere set to 15.97 % for the P1.5-1,2-2 microsphere set within the 1:300 dilution (Table 4.4). From 6.74 % for the P1.7-2,4 microsphere set to 12.39 % for the P1.5-2,10 microsphere set for the 1:900 dilution and from 3.54 % for the P1.19-1,15-11 microsphere set to 8.46 % for the P1.7-2,4 microsphere set within the 1:2700 dilution of human serum. Although these values appear to be significantly different, they are only based on a small number of replicates at low concentrations, and increasing the number of replicates used for the validation is likely to bring these values down. Possible reasons for variations between the CV values include inaccurate pipetting, splashing of reagents against the walls of the well, and temperature variations across the plate. However whilst a CV value of ≥25 is considered unacceptable, a CV value of ≤20 is considered to be a sign of good assay reproducibility, and all these values fall below this target [167].

Table 4.4 Intra-Assay Variability of replicate serum samples (n = 7) from a pool of healthy laboratory workers following immunisation with three doses of the P1.7,16 OMV vaccine. *CV was not determined as the IgG titre of anti-P1.7,16 antibodies was too high to be determined accurately at this concentration.

PorA serosubtype	CV (%) 1:300	1:900	1:2700
P1.19,15	3.82	9.46	6.53
P1.19-1,15-11	8.97	7.70	3.54
P1.5,2	14.26	7.55	7.74
P1.5-1,2-2	15.97	7.94	4.34
P1.5-2,10	6.39	12.39	6.52
P1.7,16	N/D*	9.82	4.95
P1.7-2,4	12.58	6.74	8.46
P1._,_	8.23	7.86	7.69

4.4.3.2 Inter-assay variability

Inter-assay variability was determined using three individual LiquiChip assays of human sera from healthy laboratory workers immunised with either the P1.7,16 or the P1.7-2,4 OMV vaccine. This assay was found to have low inter-assay variability. The CV of antibody concentration (Table 4.5) ranged from 1.40 % for the P1.19-1,15-11 microsphere set to 12.92 % for the P1.5-,2-2 microsphere set, for sera from healthy laboratory workers immunised with the P1.7,16 OMV vaccine. The CV of antibody concentration of sera from laboratory workers immunised with the P1.7-2,4 OMV (Table 4.6) ranged from 4.79 % for the P1.7-2,4 microsphere set to 21.86 % for the P1.19-1,15-11.

Table 4.5 Inter-assay variability shown using the arbitrary concentration and CV of three independent assays using sera from healthy laboratory workers immunised with three doses of the P1.7,16 OMV vaccine. Arbitrary antibody concentrations were determined using a standard consisting of pooled sera from healthy laboratory workers immunised with the P1.7,16 and P1.7-2,4 OMV vaccine. Concentrations calculated using 2 replicate of 6 dilutions of sera.

	PorA serosubtype							
	P1.19,15	**P1.19-1,15-11**	**P1.5,2**	**P1.5-1,2-2**	**P1.5-2,10**	**P1.7,16**	**P1.7-2,4**	**P1._,_**
Day 1	9.67	9.85	7.40	8.45	9.54	12.60	8.99	9.41
Day 2	10.00	9.71	7.80	8.44	10.77	10.85	9.23	8.68
Day 3	10.18	9.58	6.50	6.69	8.35	10.43	7.52	8.06
CV (%)	2.58	1.40	9.16	12.92	12.70	10.15	10.80	7.76

Table 4.6 Inter-assay variability shown using the arbitrary concentration and CV of three independent assays using sera from healthy laboratory workers immunised with three doses of the P1.7-2,4 OMV vaccine. Arbitrary antibody concentrations were determined using a standard consisting of pooled sera from healthy laboratory workers immunised with the P1.7,16 and P1.7-2,4 OMV vaccine. Concentrations calculated using 2 replicate of 6 dilutions of sera.

	PorA serosubtype							
	P1.19,15	**P1.19-1,15-11**	**P1.5,2**	**P1.5-1,2-2**	**P1.5-2,10**	**P1.7,16**	**P1.7-2,4**	**P1._,_**
Day 1	8.38	9.47	11.87	10.79	8.42	7.55	9.67	10.76
Day 2	10.62	11.87	10.71	10.38	10.21	8.45	9.65	9.80
Day 3	7.86	7.66	8.71	9.48	8.36	5.80	8.88	9.02
CV (%)	16.34	21.86	15.33	6.56	11.66	18.57	4.79	8.82

4.4.4 Monoplex vs. Multiplex

The ability to determine IgG concentrations of multiple anti-PorA antibodies within a single reaction would be of benefit in terms of time and volume of sera required. In order to determine whether use of a multiplex assay affected the detection of individual anti-PorA antibodies within a single reaction, serum samples were measured both in a monoplex and a multiplex assay. Pooled serum from individuals vaccinated with the P1.7,16 OMV vaccine was tested, in three dilutions in duplicate, using individual microsphere sets in a monoplex assay and a mixture of microsphere sets as a multiplex assay conducted on three different days. Arbitrary IgG concentrations were calculated using a standard curve for both the monoplex and the multiplex assay, and the CV was calculated for each set of microspheres (Table 4.7). No difference was found in IgG response to the PorA labelled microsphere, assayed using a monoplex or multiplex assay. The CV ranged from 0.01 % for the P1.5,2 microsphere set to 6.13 % for the P1.19-1,15-11 microsphere set.

Table 4.7 Comparison of monoplex and multiplex formats. Four concentrations of pooled serum samples from healthy laboratory workers immunised with three doses of the P1.7,16 OMV vaccine were tested in duplicate using the LiquiChip assay in both monoplex and multiplex formats, run on two different assays. Relative antibody concentrations were determined using a standard consisting of pooled sera from healthy laboratory workers immunised with the P1.7,16 and P1.7-2,4 OMV vaccine.

	Arbitrary IgG Concentration (ng/ml)		CV (%)
Serosubtype	**Multiplex**	**Monoplex**	**(Multi vs. Mono)**
P1.19,15	20.82	20.91	0.32
P1.19-1,15-11	19.57	21.34	6.13
P1.5,2	17.98	17.98	0.01
P1.5-1,2-2	20.47	21.97	5.03
P1.5-2,10	18.53	18.62	0.35
P1.7,16	25.94	24.42	4.25
P1.7-2,4	21.55	20.33	4.13
P1._,_	21.83	20.52	4.37

4.4.5 Assay Sensitivity and Limit of Detection.

The MFI value was recorded for the blank wells over the course of 70 assays, and the average was calculated for each microsphere set (Table 4.8). The average MFI value for the negative control sample ranged from 4.5 for the P1.5-1,2-2 microsphere set to 6.3 for the P1.5,2 microsphere set. Serial dilutions of each MAb were used to perform the Liquichip assay, and standards curves were produced, with known concentration of MAbs, as determined using the Easy-titer mouse IgG Assay kit. The lowest concentration of MAb at which background (no sera) MFI values (n = 70) were observed was calculated using the standard curve, and the limit of detection was determined as the mean MFI plus two standard deviations (SD). The limit of detection was then obtained from the standard curve. The limit of detection ranged from 41.75 pg/ml for the P1.5 MAb to 176.88 pg/ml for the P1.19 MAb (Table 4.8).

Table 4.8 Sensitivity of the LiquiChip assay. Sensitivity was estimated by determination of the highest concentration of MAb at which the MFI+ 2 SD was less than or equal to background MFI values. A mouse IgG quantification kit was used to estimate the IgG concentration of each MAb preparation. The multiplex assay was performed for a dilution series of each MAb and the antibody concentration at which background MFI values were observed was calculated.

PorA serosubtype	Background MFI	Concentration of MAb(pg/ml)
P1.19-1,15-11	5.4	176.88
P1.5-1,2-2	4.5	41.75
P1.19,15	5.4	78.25
P1.7-2,4	4.8	113.98
P1.5-2,10	5.8	55.96
P1._,_	5	42.34
P1.7,16	5.8	79.37
P1.5,2	6.3	68.80

4.5 Discussion

A multiplex LiquiChip assay was developed and used for the quantification of serum IgG responses to multiple serosubtypes of PorA in a single sample. 2500 microspheres was determined to be the optimum for each antigen to be tested. This is in agreement with other microsphere based multiplex assays[242]. This provided the greatest dynamic range of the assay, defined as the range over which there is a linear relationship between antibody concentration and MFI. Differences in the IgG response determined using different numbers of microspheres may be due to saturation of the microspheres with antibody. With a constant amount of serum antibody, microsphere saturation with antibodies will decrease as the number of microsphere increase and may result in microspheres that have not been bound to antibodies. As MFI was calculated as an average of 100 microspheres, this will be affected by the presence of microspheres not bound to anti-PorA antibodies and the MFI will be reduced. Conversely, as the number of microspheres is reduced, saturation of the microspheres with anti-PorA IgG will be increased. Once all the anti-PorA binding sites on the microspheres have been saturated, the maximum MFI has been reached and will not be affected by the presence of unbound antibodies in the serum samples. Therefore, use of this number of microsphere allows for the assay to be performed at an increased range of concentrations reducing the need for sera to be retested at different concentrations. This wide dynamic range should lead to a reduction in the number of sample dilutions required, whilst the accuracy of the assay is increased and lower levels of antibody can be detected[167].

The use of a multiplex assay was not found to compromise the results of this assay, and no evidence of interference between microsphere sets was detected. Similar concentrations were measured for anti-PorA IgG concentrations using the monoplex and multiplex assay confirming suitability of the Liquichip assay to detect IgG responses to multiple PorA serosubtypes simultaneously. Assay reproducibility was demonstrated with low CV values calculated for both inter- and intra-assay variability similar to those reported for assays using other antigens[167,168,67,329,275].

The limit of detection, the lowest level of antibody response that can be detected, as opposed to a true negative value was determined using MFI values from the negative control sample plus two SD and the corresponding MAb concentration at which this MFI was recorded. The assay was found to be highly sensitive with the lowest detectable concentration ranging from 42.75 - 176.88 pg/ml. In comparison, the lowest concentration of anti-PorA IgG detected using ELISA has been reported as ≤2 μg/ml[128,343] and an ELISA for the detection of IgG directed to the P64K protein of *N. meningitidis* reported a limit of quantification validated to 1.25 ng/ml[175]. Whilst no direct comparison between ELISA and the multiplex assay were performed in this study, previously studies have shown the multiplex assay to be more sensitive than ELISA[242,325], although good correlation between IgG concentrations measured using the ELISA and multiplex assay has been observed[167]. Multiplex assays based on the detection of anti-capsular antibodies have been shown to be less specific than the anti-PorA assay with a range of 370 to 650 pg/ml observed for anticapsular antibodies quantified using a tetraplex assay[167]. The chemical composition of antibodies may be a cause of the differing sensitivity, with marked differences produced in immune responses to polysaccharide vaccines and protein vaccines as stated in Chapter 1.

MAbs directed to a single VR of the PorA protein were used to demonstrate the assay was specific for the detection of anti-PorA IgG responses with cross-reactivity only observed between anti-PorA antibodies and microspheres labelled with variants of the same PorA family, correlating with published results on the specificity of these MAbs[2,348,239,344]. Assay specificity was further demonstrated using inhibition of MAb binding with purified proteins, and inhibition was only observed in the binding of MAbs specific to the PorA serosubtype of the inhibiting protein. For the majority of the PorA labelled microsphere sets, complete inhibition of MAb binding with the corresponding PorA protein was observed, whilst limited inhibition was observed in others. There are several possible explanations for this limited inhibition including: (i) a high concentration of MAbs and insufficient protein to ensure complete binding of antibodies; (ii) antibody affinity influencing the effect of inhibiting protein; and (iii) competition between binding sites on the free protein and the microsphere conjugated protein. As PorA has two VRs, inhibition of binding to

one of the VRs does not inhibit antibody binding to the second VR[198] resulting in partial inhibition of the IgG response.

Whilst the specificity of the assay was clearly demonstrated with the use of mouse MAbs, produced in response to a specific PorA serosubtype, a different response was measured using the more complex human sera. Carriage of meningococci in humans and the resulting immune response has been well documented[114,356]. The presence of pre-existing antibodies corresponding to multiple PorA serosubtypes in human serum would therefore be expected, with immune responses directed to one or both of the two VRs produced in response to either vaccination or carriage[324]. Whilst pre-incubation of human serum with PorA resulted in decreased antibody binding to the corresponding protein labelled microspheres, a decrease in binding response was also observed with unrelated PorA serosubtypes. It is possible pre-existing antibodies in serum may be cross-reacting with the purified protein used for inhibition. Alternatively the presence of antibodies in serum may be cross-reacting to the protein labelled microsphere. The P1.19,15, P1.19-1,15-11 and the P1.5-2,10 proteins had the greatest inhibitory effect on all the microspheres sets tested. All of these had a shared amino acid sequence in loop 6 (Figure 1.4) that differed from the deduced amino acid sequence of the other PorA proteins used in this assay and may be a potential site for cross reactivity. The P1.5 MAb was not found to bind to the P1.5-2 epitope of purified PorA protein however some cross-reaction between the serosubtype P1.5-2,10 purified PorA protein and the serum antibodies was detected against the P1.5 and the P1.5-1 epitope. This suggests the possibility of serum antibodies binding to parts of the protein outside of the epitope recognised by the MAb. A possible explanation for the limited inhibition of P1.7,16 and the P1.7-2 MAb, is that P1.7-2 PorA serosubtype has been shown to have a hidden epitope that may not be available for antibody binding using whole cell ELISA[345]. This is due to a deletion in the P1.7 epitope causing movement of the epitope towards the outer membrane where it is masked by the other loops. The presence of this hidden epitope may also explain the inhibition observed between the P1.7 MAb and P1.7-2,4 labelled microspheres[345]. Another possible explanation for the limited inhibition in binding observed between the P1.7 MAb

and the P1.7-2,4 labelled microspheres may be explained by competition between the P1.7 and the P1.7-2 epitopes. The P1.7 MAbs are more weakly bound to the P1.7-2 epitope than they are to the P1.7 epitope, and so are more likely to be outcompeted following inhibition with the P1.7-2,4 protein. Although it is more likely that the IgG response measured for this protein is due to binding of MAbs corresponding to the VR2 region of the protein. Similarly the P1.15 antibody has a higher affinity for the P1.15 epitope than the P1.15-11. In order to determine antibody affinity to specific epitopes, a competition assay would need to be performed with titration of a competitor. MAbs are mouse antibodies produced in response to a human antigen, and monoclonal antibodies may produce a differing response to human sera antibodies. Reactivity of these antibodies are also determined using ELISA, and the MAbs may respond differently in a Liquichip assay.

It is important to note that the standard used for the calculation of all human sera IgG responses in this assay, was composed of sera collected from individuals vaccinated with three doses of either the P1.7,16 or the P1.7-2, 4 OMV vaccine. Difficulties arise with the use of pooled human sera from vaccinated laboratory workers as a standard, due to unknown concentrations of antibodies to different PorA serosubtypes. Each serosubtype specific IgG response produced by the standard was assigned the same arbitrary concentration, resulting in a serosubtype specific anti-PorA antibody with a low MFI value, assigned the same relative concentration as one with a high MFI value. As serum antibody levels were quantified relative to the respective PorA specific antibody concentration in a standard serum, whilst the concentration of antibodies directed to the same PorA serosubtype can be compared over several assays, direct comparison cannot be made for IgG responses and antibody concentrations between different serosubtypes. Additionally, whilst assay sensitivity can be measured using mouse MAbs, it is not possible to measure assay sensitivity using human sera. However, this can be improved with the use of a suitable reference serum consisting of known concentrations of human meningococcal antibodies. Production of a human standard with known concentrations of serosubtype specific anti-PorA would aid in comparisons of anti-PorA titres across differing serosubtype within the

same assay, and comparison of this assay with similar assays. Unfortunately, no reference standard is available for this purpose and would be very difficult to produce due to the complex nature of human adult sera. Infant sera, with no previous exposure to meningococcal isolates are unlikely to have cross reacting antibodies, and post-vaccination sera would be ideal for use as a human standard. However due to the limited volumes of infant sera available, this is not a feasible option.

The Liquichip assay described in this chapter was optimised using mouse MAbs and human sera and was found to be specific, sensitive and reproducible. There are several advantages to the use of a multiplex assay such as the one described in this chapter, including a greater dynamic range over which the assay can be performed, and increased sensitivity in comparison to the traditional ELISA. The multiplex nature of this assay allows for the detection of antibodies directed to eight serosubtypes of PorA simultaneously, thereby saving both time and volume of sera required for the assessment of immune responses. This would be of benefit for the evaluation of immune response resulting from immunisation with multicomponent vaccines and the use of multiplex assays to enhance preclinical evaluation of immune responses has previously been described for meningococcal capsular polysaccharides, diphtheria, tetanus toxin, *Hib* capsular polysaccharide and ***Bordetella pertussis*** antigens [230,167,168,242,285]. Another advantage of the liquichip assay is the potential ability to extend the assay to quantify additional serosubtypes of PorA or alternative antigens, such as the PorB, FetA and fHbp proteins described in the previous chapter.

Chapter 5. Analysis of human and mouse sera using the PorA Liquichip assay following vaccination with outer membrane vesicle vaccines.

5.1 Introduction

Availability of capsule based vaccines have contributed to the prevention of meningococcal disease caused by four of the five major-disease causing serogroups of *N. meningitidis*[314]. However, disease caused by serogroup B meningococci continues to be a problem, due to poor immunogenicity of the serogroup B capsule and immunological similarity to host sialic acids[354,93]. OMV vaccines have been shown to confer immunity and can be used to control the incidence of serogroup B disease, however these tend to be limited to specific epidemic strains, such as the Norwegian based P1.7,16 OMV vaccine (MenBvac), and the New Zealand based P1.7-2,4 OMV vaccine (MeNZB)[27,191,274,216]. Vaccines such as these afford little cross protection against antigenically diverse meningococcal strains[295,349]. This lack of cross protection is due to the variability of the major immunodominant proteins such as PorA and Opc present in OMV vaccines[253]. In order to overcome the limited coverage of OMV vaccines based on a single serosubtype of PorA, vaccines based on two or more OMVs, each containing multiple PorA proteins have been engineered[42]. Further development of vesicle vaccines include incorporation of purified subcapsular antigens or multivalent formulations such as the Hexamen (6-valent) or Nonamen (9-valent) OMV vaccines[42,108,316,259].

The evaluation and comparison of immune responses to meningococcal vaccines have classically been performed using two basic groups of laboratory assays: antibody binding assays and functional assays. Antibody binding assays provide a good indication of immune responses to vaccination, but only an indirect measure of protective immunity. Antibody binding assays such as the ELISA detect both functional and non-functional antibodies, with antibodies of lower avidity giving poor correlation with functional assays. Modified ELISAs have been developed for the detection of high avidity antibodies. Functional assays such as the SBA assay predominantly measure high-avidity antibodies shown to be superior to low avidity antibodies for bactericidal

activity[238]. The ability of SBAs to confer protection against meningococcal disease is widely accepted, and the presence of SBAs is generally considered the best indicator of protective immunity[113]. For purposes for licensure of meningococcal vaccines, regulatory agencies generally accept induction of these antibodies as evidence of vaccine effectiveness[32,207]. The SBA assay has been evaluated as the correlate of protection for OMV vaccines, and is primarily used for the determination of immunogenicity of OMV vaccines[189,216,92], with a relationship between SBA and anti-PorA antibodies noted[27,253,349].

5.2 Aims of this chapter

The aims of this chapter were:

- To assess the ability of the multiplex PorA assay to measure increases in binding antibody titres to specific PorA serosubtypes induced by vaccine candidates in preclinical immunogenicity trials;
- To assess the ability of the multiplex PorA assay to measure increases in antibody binding titres to specific PorA serosubtypes induced by vaccine candidates in clinical immunogenicity trials.

5.3 *Source of Sera used*

Sera collected as part of four separate vaccine trials were analysed in this chapter (Table 5.1). Sera samples obtained from the RIVM, The Netherlands, were collected as part of the Hexamen or the Nonamen vaccine trial. These studies were performed to determine reactogenicity and immunogenicity of the two OMV vaccines. Following vaccination with the Hexamen vaccine, ELISA and bactericidal assays were used to demonstrate serosubtype specific responses in mice, with antibody responses observed against all serosubtypes of PorA included in the vaccine. The magnitude of antibody responses was not found to be equal across all PorA serosubtypes. The highest titre was raised against the P1.5-1,2-2 serosubtype of PorA, followed by the P1.7,16 serotype. The lowest immune responses were found to be induced against the P1.19,15-1 and

P1.7-2,4 serosubtypes. Similar results were observed for the production of bactericidal antibodies with 12-fold higher titres observed against the P1.5-1,2-2, P1.5-2, 10 and P1.7,16 serosubtypes of PorA than the P1.7-2,4, P1.19,15-1 and P1.12-1,13 serosubtypes of PorA[319,323,50,182]. Immune responses to the nine PorA serosubtypes in the Nonamen vaccine were also found to vary in mice, with the highest SBA responses determined against the P1,7,16, P1.5-1,2-2, P1.5-2,10 and the P1.22,14 serosubtypes of PorA. The lowest SBA response was again determined against the P1.19,15-1 serosubtype of PorA[64,63,316].

Murine sera was collected following immunisation with two injections of 1/10 human dose of either Hexamen or Nonamen vaccine. Human sera were collected following immunisation of healthy Netherlands toddlers of 2 - 3 years of age, and children of 7 – 8 years of age with three injections of the Hexamen vaccine[64,63,316].

Sera from the SEU were collected following vaccination of healthy laboratory workers with OMV vaccines based on single strains of serogroup B meningococci as part of two NVEC studies[92]. Laboratory workers were immunised with three doses of either the P1.7,16 or the P1.7-2,4 OMV vaccines, and pre- and post-vaccination sera were collected. Unfortunately, SBA data were not available for any of the sera samples described in this chapter, and could not be used for correlation with data collected using the Liquichip assay.

Table 5.1 Source of sera used in this chapter.

Source	Serum	Sera Samples
RIVM (The Netherlands)	Hexamen OMV vaccine	32 samples (Murine)
		11 samples (Human)
	Nonamen OMV vaccine	32 samples (Murine)
SEU (Manchester)	NW P1.7,16 OMV vaccine (MenBvac)	17 samples (Human)
	NZ P1.7-2,4 OMV vaccine (MeNZB)	9 samples (Human)

5.4 Results

5.4.1 Analysis of murine sera following vaccination with either Hexamen (6 - valent) or Nonamen (9 - valent) OMV vaccine

Serum samples from mice immunised with either the Hexamen or the Nonamen vaccine were obtained from the RIVM and analysed using the PorA Liquichip assay developed in chapter four. Similar IgG responses were detected following immunisation with both the Hexamen and the Nonamen vaccines, with no significant difference in the geometric mean titre (GMT) for antibodies directed to any of the PorA serosubtypes tested. The lowest GMT was determined using the P1.7,16 labelled microspheres and the greatest responses directed towards the P1.5-2,10 and the P1.7-2,4 serosubtypes (Figure 5.1). IgG responses could not be detected for the unvaccinated control group to any of the PorA labelled serosubtypes, with the exception of P1.7-2,4 for which a low GMT was detected.

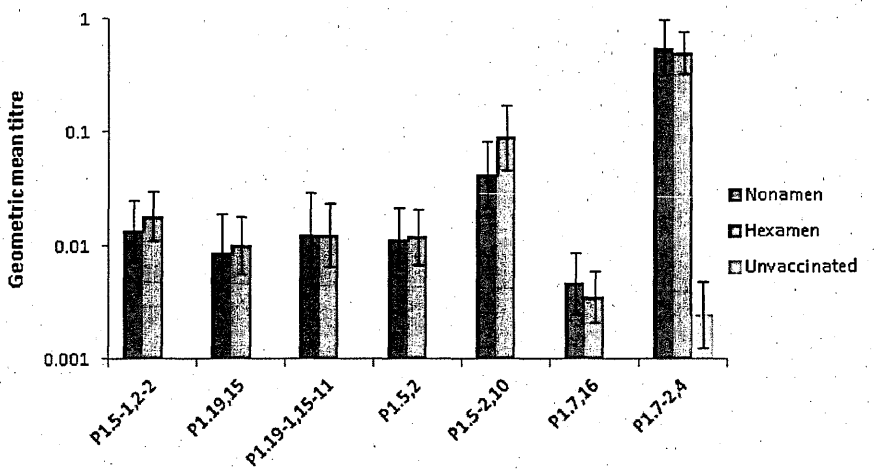


Figure 5.1 Geometric mean IgG titres of murine sera vaccinated with Nonamen or Hexamen OMV vaccine and an unvaccinated control group (error bars represent 95 % confidence intervals). Samples were obtained from Balb/c mice immunised with 1/10 human dose of vaccine (1.5 µg/PorA) on days 0 and 28 with blood taken on day 42. Data were based on n = 32 with the exception of the P1.5-2,10 serosubtype, where n = 30 and n = 31 for Nonamen and Hexamen, respectively, and for the P1.7-2,4 serosubtype, where n = 31 for the Nonamen vaccinated group. All samples were run in duplicate within a single experiment. Titres were determined using a standard composed of a mixture of mouse monoclonal antibodies.

5.4.2 Analysis of human toddler and child sera following vaccination with Hexamen OMV vaccine

Serum samples from healthy toddlers (2 – 3 years old) and children (7 – 8 years old) immunised with the Hexamen OMV vaccine were obtained from the RIVM and analysed using the PorA Liquichip assay (Figure 5.2). Samples consisted of one pre-vaccination sample, two post-primary samples (following immunisation with the second dose of the vaccine) and seven post-boost samples (following immunisation with the third dose of vaccine). None of these samples were from the same individual. An adult serum sample was used as a positive assay control, as this sample was known to have high levels of anti-PorA antibodies. Sample selection was based purely on availability of samples, as all samples and the positive assay control were kindly donated by the RIVM for use in this study. Similar antibody titres were observed against all serosubtypes used in this assay, with low IgG titres observed for the pre-vaccination and post-primary samples in comparison to the post-boost samples. In particular, for GMTs determined against the P1.5,2, P1.5-1,2-2 and P1.5-2,10 and P1.7,16 serosubtypes, the effect of the first dose of vaccine was minimal, with similar titres determined for both post-primary sera and pre-vaccination serum. Clear differences between the two post-primary samples were evident. Lower IgG concentrations were determined against all PorA labelled microspheres for post-primary 1 serum than for the pre-vaccination serum with the exception of the VR1 and VR2 deleted PorA mutant, P1._,_. In contrast, sera from post-primary individual 2 demonstrated a higher antibody response to all PorA serosubtypes with the exception of the P1.5,2 and the P1.5-2,10 serosubtypes, where a lower response was observed in relation to pre-vaccination serum (Table 5.2). In particular, IgG concentrations determined against the P1.19,15; P1.19-1,15-11; P1.7-2,4 serosubtypes and the PorA P1._,_ mutant were shown to exhibit a greater than two-fold higher response in relation to pre-vaccination serum.

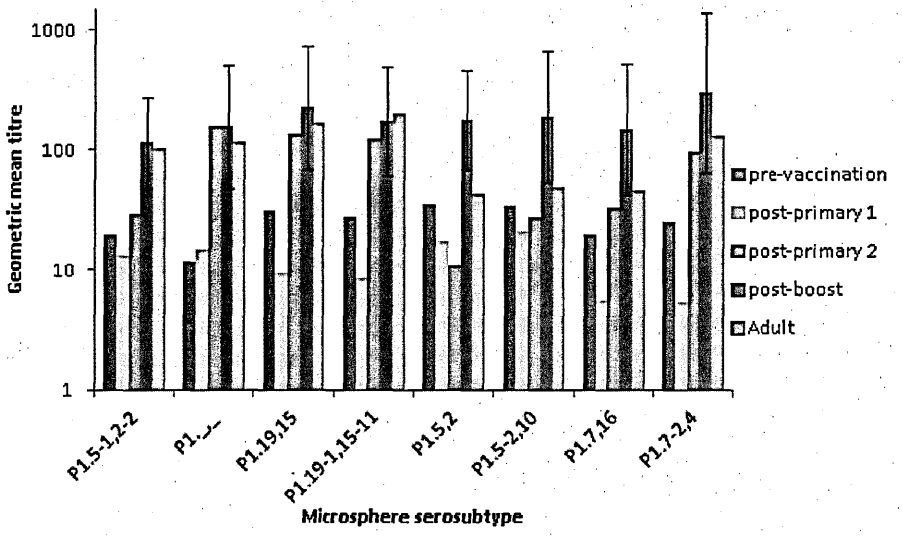


Figure 5.2 Geometric mean IgG titres of sera from healthy toddlers (2 – 3 years old) and children (7 – 8 years old) vaccinated with the Hexamen OMV vaccine. An adult serum sample was included as a positive assay control (error bars represent 95 % confidence intervals). The vaccine was administered at 0, 2, and 8 months. Pre-vaccination serum was taken prior to the first dose, whilst post-primary and post boost sera was taken four - six weeks following the second and third dose of vaccine, respectively. The pre-vaccination and adult serum response are representative of single individuals, post-primary sera responses of two individuals (shown separately) and the post boost sera responses are the GMT of seven individuals with the exception of P1.5-1,2-2, P1._,_, and P1.5,2 serosubtypes where n = 6 and P1.5-2,10 serosubtype where n = 5. All samples were run in duplicate within a single experiment and titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with the P1.7,16 or the P1.7-2,4 OMV vaccine.

The largest IgG titres, determined against all serosubtypes were observed for the post-boost samples, with a greater than two-fold higher response observed in GMTs for all serosubtypes in relation to the pre-vaccination sample. The IgG titres for the adult sample were found to be higher than those observed for the pre-vaccination sample for all PorA serosubtypes, with a greater than two-fold higher response in titre determined against the P1.5-1,2-2; P1.19,15; P1.19-1,15-11; P1.7-2,4 serosubtypes and the P1._,_ PorA mutant.

The Liquichip assay is used to determine the presence of antigen binding antibodies, it is not a measure of functional bactericidal activity. The detection of antigen binding antibodies does not

confirm a protective immune response, and demonstration of bactericidal antibody responses would need to be confirmed with use of the SBA assay. This data would be of particular importance in the evaluation of vaccines, as the SBA assay is considered to be the gold standard for determining meningococcal vaccine efficacy, and is used to confer protection against meningococcal disease. Data from SBA assays has been found to show good correlation with antigen binding antibody assays, such as the ELISA). In particular, good correlation was observed using sera from immunised teenagers and adults following immunisation with a single vaccine [10]. SBA data was not available for the sera used in this experiment.

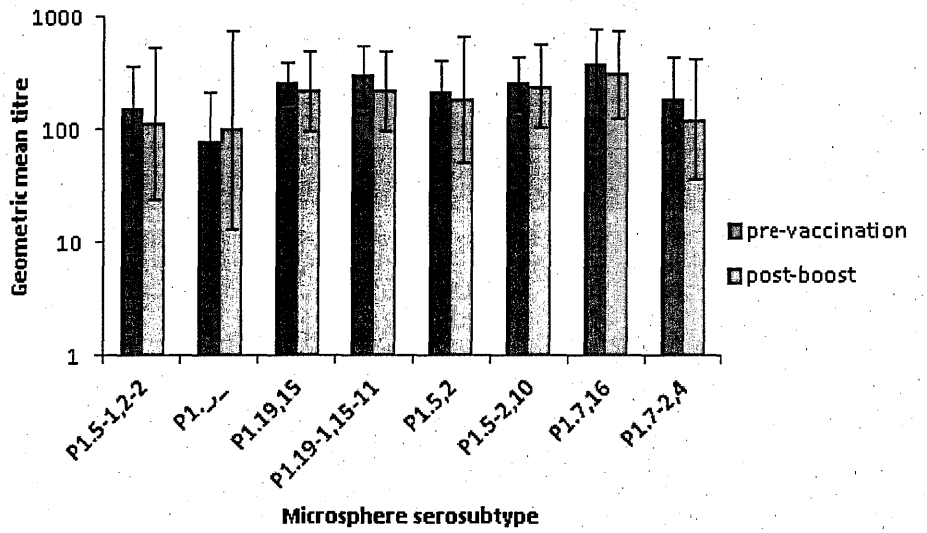
Table 5.2 Fold higher responses in arbitrary antibody titre observed following vaccination with Hexamen OMV vaccine. Numbers in bold represent a greater than two-fold higher response relative to the pre-vaccination serum sample. Adult control and post-primary IgG responses are representative of single individuals and post boost sera responses are the geometric mean of seven individuals with the exception of the P1.5-1,2-2, P1._,_, and P1.5,2 serosubtypes where n = 6 and P1.5-2,10 serosubtype where n = 5. All samples were run in duplicate within a single plate.

Sample	Serosubtype							
	P1.5-1,2-2	**P1._,_**	**P1.19,15**	**P1.19-1,15-11**	**P1.5,2**	**P1.5-2,10**	**P1.7,16**	**P1.7-2,4**
Post-primary 1	0.67	1.27	0.31	0.31	0.51	0.61	0.28	0.21
Post-primary 2	1.52	**13.59**	**4.44**	**4.48**	0.32	0.80	1.67	**3.90**
Post-boost	**5.88**	**13.49**	**7.34**	**6.29**	**5.17**	**5.50**	**7.63**	**12.02**
Adult control	**5.25**	**10.14**	**5.42**	**7.28**	1.26	1.42	**2.37**	**5.30**

5.4.3 Analysis of human sera following vaccination with OMV vaccines

The effect of vaccination on PorA specific IgG concentrations was evaluated using sera from healthy human adults developed against either the P1.7,16 or the P1.7-2,4 OMV vaccine. Pre- and post-vaccination sera were analysed using the PorA Liquichip assay and IgG titres were determined against the PorA labelled microsphere panel. A standard curve was used to quantify the antibody response in human sera with an arbitrary concentration assigned for each PorA specific IgG response determined against each of the PorA serosubtypes included in the assay as described earlier in this thesis.

5.4.3.1 Analysis of human adult sera following vaccination with the P1.7,16 OMV vaccine



P1.5-1,2-2	P1._,_	P1.19,15	P1.19-1,15-11	P1.5,2	P1.5-2,10	P1.7,16	P1.7-2,4
0.74	1:28	0.84	0.74	0.84	0.96	0.82	0.66

Figure 5.3 Geometric mean IgG titres of sera from healthy laboratory workers vaccinated with the P1.7,16 OMV vaccine (error bars represent 95 % confidence intervals). Pre-vaccination sera were collected prior to administration of the first dose, post-vaccination (boost) sera were taken six weeks following the third dose of vaccine. Responses are shown as the GMT of 17 individuals with the exception of P1.5-2,10, where n=16 for the pre-vaccination sample. Samples were run in duplicate at two dilutions in order to ensure antibody concentrations were within the linear range of the assay. Differences in GMTs between pre- and post-vaccination sera are shown. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

Sera from seventeen healthy adults were collected both prior to vaccination and six weeks after administration of the third dose of the P1.7,16 OMV vaccine. Paired samples were analysed and GMTs were calculated for both pre- and post-vaccination sera. Similar GMTs were observed for all samples tested (Figure 5.3), with slightly reduced titres in response to the P1._,_ labelled microspheres. Although high titres were observed, no significant difference was observed between the pre- and post-vaccination sera samples and may be due to pre-existing antibodies present in the samples. Unfortunately, SBA data were not available for these serum samples, and could not be used for correlation with data produced using the Liquichip assay.

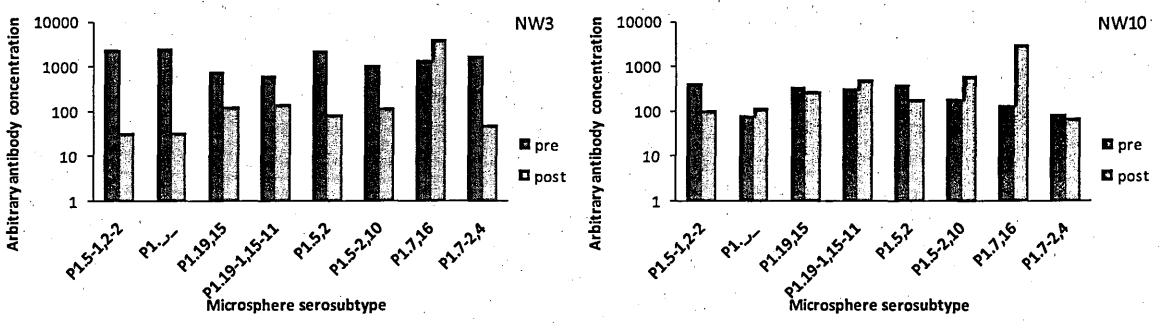


Figure 5.4 Arbitrary antibody titres from two individuals showing a specific increase in the titre of P1.7,16 antibodies. Pre-vaccination sera were taken prior to administration of the first dose and post-vaccination (boost) sera were taken six weeks following the third dose of the P1.7,16 OMV vaccine. Each sample was run in duplicate at two dilutions in order to ensure antibody concentrations were within the linear range of the assay. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

The presence of both high responders, exhibiting increased titres to all serosubtypes, and low responders, exhibiting unchanged or decreased IgG titre to all serosubtypes were evident when sera were analysed individually. Pre- and post-vaccination titres were determined against all PorA labelled microsphere sets for individuals. Only two individuals, NW3 and NW10, exhibited a specific increase in IgG titre determined using the P1.7,16 PorA labelled microspheres (Figure 5.4). A decrease in IgG titre was observed against all other PorA serosubtypes following vaccination of

individual NW3 with the P1.7,16 OMV vaccine, with the exception of the P1.7,16 serosubtype, for which a 2.9-fold increase was found. IgG titres in sera from individual NW10, were found to be largely unaffected following vaccination with the exception of the P1.7,16 serosubtype against which a 23-fold increase was measured. An increase was also observed against the unrelated P1.5-2,10 serosubtype of PorA with a 3.3-fold increase in IgG titre.

Several individual were found to be positive responders with an increase in IgG titre observed against all PorA serosubtypes included in the Liquichip assay (Figure 5.5). Whilst the magnitude of response varied between individuals, the largest increase in titre for all individuals, with the exception of NW17, was determined using the PorA P1._,_ mutant. Difference in titres determined against the P1._,_ labelled microspheres may be misleading, due to the low concentration of antibodies able to bind to this protein in the human standard. Use of arbitrary concentrations result in low levels of antibodies assigned the same concentration as antibodies present in much higher concentrations. A small difference in MFI value could therefore result in a large difference in arbitrary concentration for this microsphere set. The increase in IgG titre for Individual NW13 ranged from 1.38 to a maximum of 4.0 fold determined against the P1.5-2,10 and P1._,_ PorA mutant labelled microspheres, respectively. Individual NW21 exhibited a slight decrease in IgG titre determined against the P1.5,2 and the related variant P1.5-1,2-2 labelled microspheres. An increase was observed against all other serosubtypes and ranged from 1.48 to 12.03 fold determined against the P1.5-2,10 and the PorA mutant, respectively. Individual NW2 was found to be a particularly high responder with significant increases in IgG titre determined against the P1.5-1,2-2; P1.5,2 and P1.7-2,4 serosubtypes of PorA with 139, 88.67 and 97.94 fold increases observed, respectively. IgG titres determined against P1.19,15 and the related P1.19-1,15-11 labelled microsphere were not affected by vaccination for this individual. Individuals NW7 and NW17 were determined to have elevated IgG titres following vaccination against all serosubtypes of PorA analysed in this assay. The smallest increase in IgG titre for NW7 was determined against the P1.7,16 labelled microspheres whilst the largest increase was again determined against the P1._,_ PorA mutant labelled microsphere set. Serum from individual

NW17 was shown to have elevated IgG titres against all the PorA serosubtypes, with the smallest 4.13 fold increase determined against the P1.5-2,10 labelled microspheres. Unlike the other high responders, the largest increase in IgG titre for Individual NW17 was determined against the P1.7-2,4 labelled microspheres with a 29.42 fold increase. The highest IgG titres were observed in serum collected from individual NW14, with increases ranging from 4.27 to 417 fold determined against the P1.7,16 and PorA P1._,_ mutant labelled microspheres, respectively. These sera have been collected from adult laboratory workers and as such are likely to have had previous natural exposure to meningococcal isolates, confirmed by the presence of serosubtype specific anti-PorA antibodies observed in pre-vaccination sera. The vaccine may be acting to boost a pre-existing immune response, and use of this vaccine has previously been reported to induce significant increases in IgG responses to homologous and heterologous PorA antigens[342]. SBA assay data for these sera would be of value in determining whether the production of bactericidal antibodies had been induced by this vaccine.

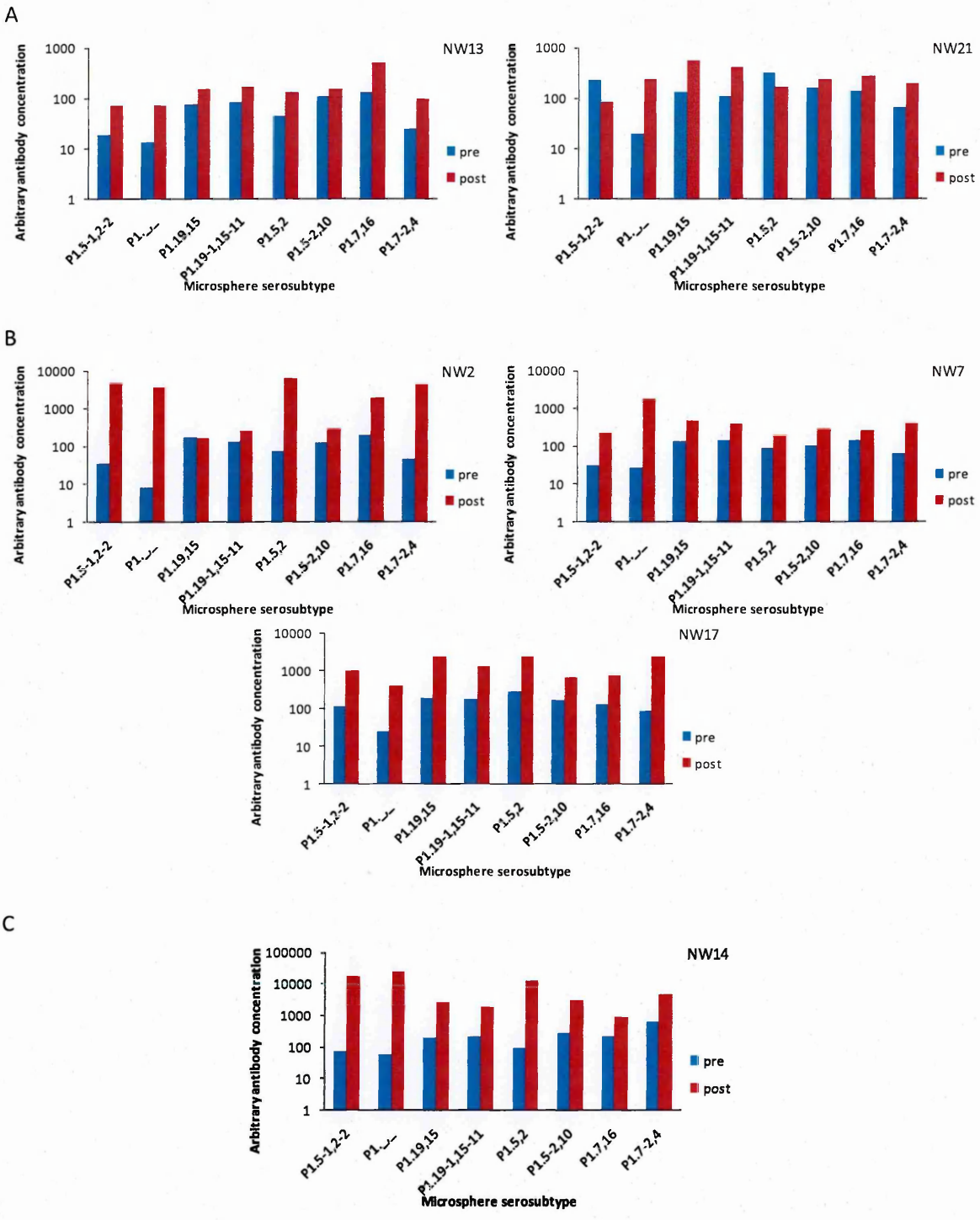


Figure 5.5 Arbitrary antibody titres from individuals found to have increased IgG titres to all PorA serosubtypes following immunisation with the P1.7,16 OMV vaccine. Pre-vaccination sera were taken prior to administration of the first dose, post-vaccination (boost) sera were taken six weeks following the third dose of vaccine. Each sample was run in duplicate at two dilutions in order to ensure antibody concentrations were within the linear range of the assay. Individuals are grouped into differing magnitudes of IgG titres, shown as A, B and C. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with the P1.7,16 or the P1.7-2,4 OMV vaccine.

The majority of individuals vaccinated with the P1.7,16 OMV vaccine were found to be non-responders when sera were analysed using the Liquichip assay. Post-vaccination sera from eight individuals were found to have unchanged or decreased IgG titres determined against all PorA labelled microspheres in relation to pre-vaccination sera (Figure 5.6). IgG titres in sera collected from individuals NW6, NW8 and NW11 were not found to be affected following vaccination with a P1.7,16 OMV vaccine, with a less than twofold difference determined against any of the PorA labelled microspheres. Decreases in IgG titre were determined to all PorA labelled microsphere for individuals NW4, NW12, NW16, NW18 and NW20 following vaccination with three doses of the P1.7,16 OMV vaccine. As previously stated, the likelihood of pre-existing antibodies directed against PorA serosubtypes representative of those prevalent in the UK is increased with the use of adult human sera due to prior natural exposure. High pre-vaccination titres of anti-PorA antibody in the pre-vaccination may therefore result in the vaccine appearing to have little or no effect.

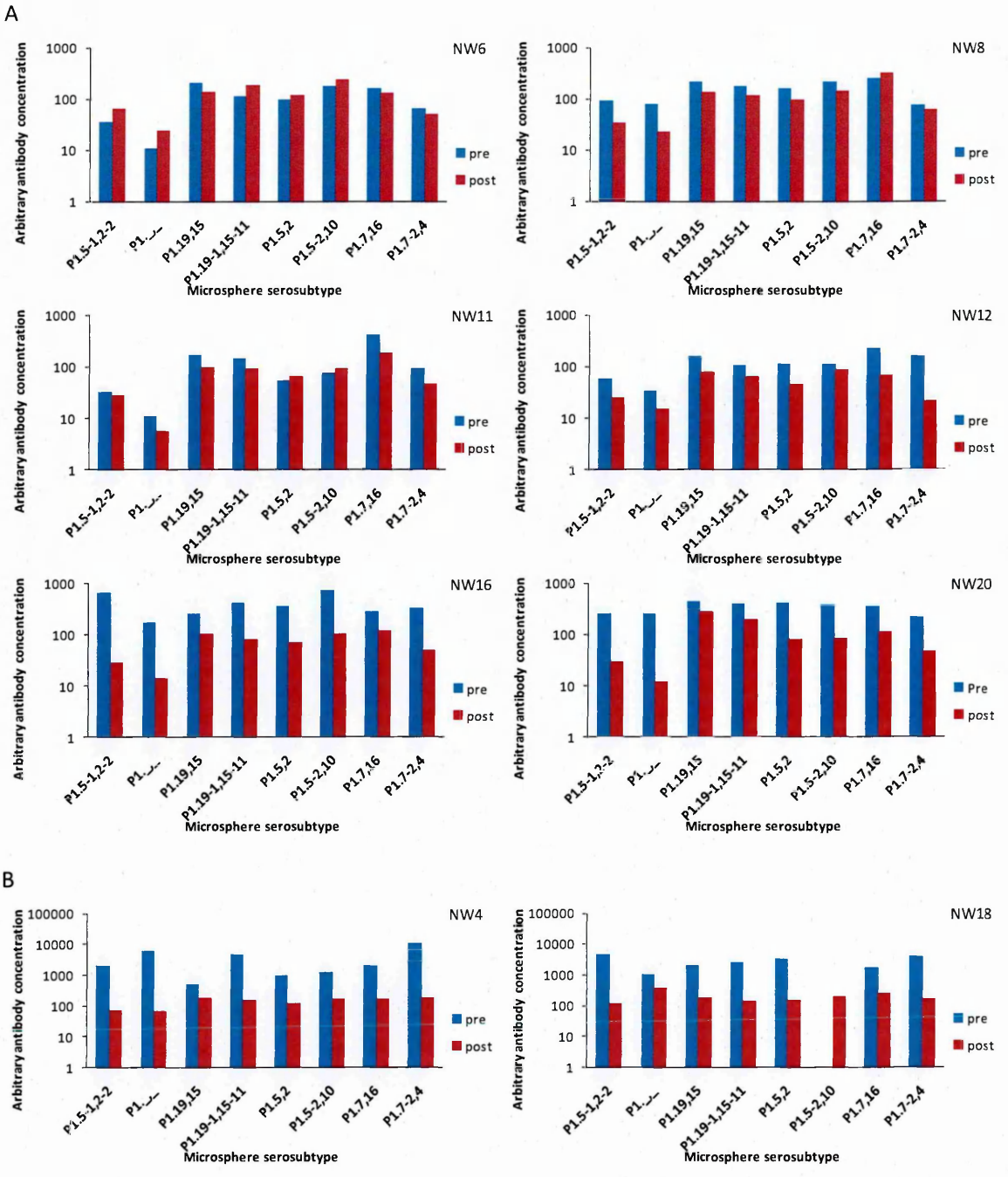
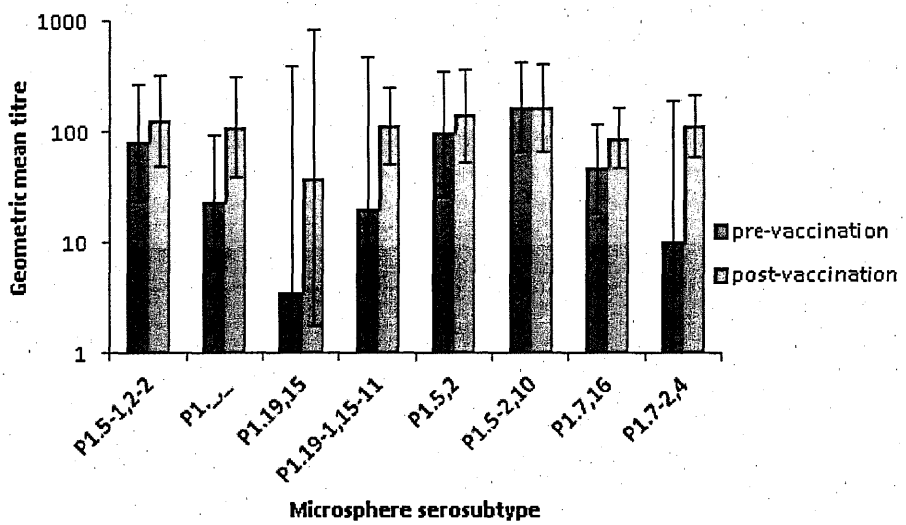


Figure 5.6 Arbitrary antibody titres from individuals determined to be non-responders using the Liquichip assay, with decreased IgG titres observed following immunisation with the P1.7,16 OMV vaccine. Pre-vaccination sera were collected prior to administration of the first dose, post-vaccination (boost) sera were taken six weeks after the third dose of the P1.7,16 OMV vaccine. Each sample was run in duplicate at two dilutions in order to ensure antibody concentrations were within the linear range of the assay. Individuals are grouped into differing magnitudes of IgG titres, shown as A and B. The pre-vaccination IgG titre of NW18, was not determined against the P1.5-2,10 labelled microspheres. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with the P1.7,16 or the P1.7-2,4 OMV vaccine.

5.4.3.2 Analysis of sera from healthy human adults following vaccination with the P1.7-2,4 OMV vaccine



P1.5-1,2-2	P1._,_	P1.19,15	P1.19-1,15-11	P1.5,2	P1.5-2,10	P1.7,16	P1.7-2,4
1.57	**4.74**	**10.97**	**5.56**	1.46	0.99	1.87	**11.44**

Figure 5.7 Geometric mean IgG titres of sera from healthy adults vaccinated with the P1.7-2,4 OMV vaccine (error bars represent 95 % confidence intervals). Numbers in bold represent a greater than two fold increase in titre following vaccination. Pre-vaccination sera were taken prior to the first dose, and post-vaccination sera were collected six weeks after third dose of the P1.7-2,4 OMV vaccine was administered. Each sample was run in duplicate at two dilutions in order to ensure antibody concentrations were within the linear range of the assay. Responses are shown as the GMT of 9 individuals for both pre- and post-vaccination sera. Fold increases in GMT observed following vaccination are shown in relation to pre-immunisation samples. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

Sera was collected from nine healthy laboratory workers both prior to vaccination and six weeks after administration of the third dose of the P1.7-2,4 OMV vaccine. Paired samples were analysed using the Liquichip assay and GMTs were calculated for both pre- and post-vaccination sera. In contrast to results obtained with sera following vaccination with the P1.7,16 OMV vaccine, increased GMTs were determined against all PorA serosubtypes following vaccination with the P1.7-2,4 OMV vaccine, with the exception of the P1.5-2,10 labelled microsphere set (Figure 5.7).

The greatest increases in antibody titre were determined against the PorA P1._,_ mutant; P1.19,15; P1.19-1,15-11 and P1.7-2,4 labelled microsphere sets, all of which revealed a greater than two fold increase in the GMT of post-vaccination sera in comparison to pre-vaccination sera. A more limited increase was observed against the P1.7,16 labelled microspheres and the unrelated P1.5,2; P1.5-1,2-2; and P1.5-2,10 serosubtypes. The P1._,_ is a PorA mutant expressed as a PorA protein without the VR1 and VR2 antigenic binding epitopes. Bactericidal antibodies directed against the VR3 region of PorA have been produced, and any antibody titres determined against the P1._,_ -PorA protein are likely to be directed against this region.

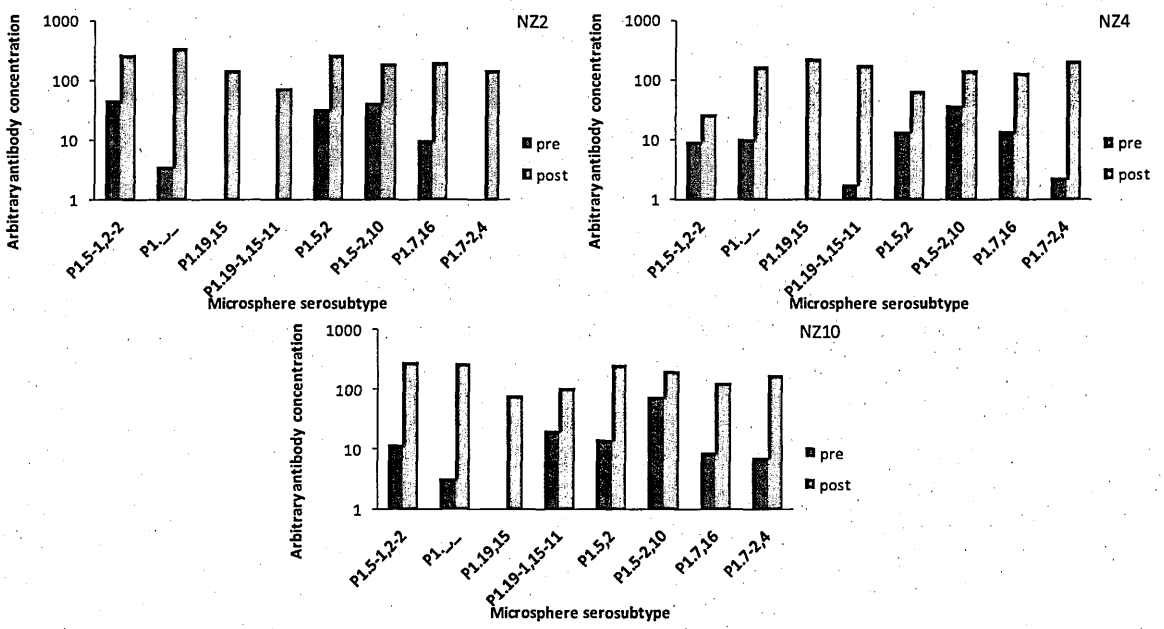


Figure 5.8 Arbitrary antibody titres from three individuals showing increased IgG titres determined against all PorA labelled microspheres following vaccination with the P1.7-2,4 OMV vaccine. Pre-vaccination sera were taken prior to administration of the first dose, post-vaccination (boost) sera were collected six weeks after the third dose of the P1.7-2,4 vaccine. Each sample was run in duplicate at two dilutions in order to ensure antibody concentrations were within the linear range of the assay. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

The effect of vaccination was again found to be dependent on the individual, with both high and low responders to the vaccine observed when sera was analysed individually. Individuals NZ2, NZ4 and NZ10 were found to have increased IgG concentrations determined against all panel

PorA labelled microspheres following vaccination with the P1.7-2,4 OMV vaccine (Figure 5.8). IgG titres determined against the P1.19,15 labelled microspheres for all these individuals were found to be below the limit of detection in pre-vaccination sera. Pre-vaccination sera from individual NZ2 was also found to have IgG titres below the limit of detection using microspheres labelled with serosubtype P1.19-1,15-11 and P1.7-2,4 purified PorA proteins.

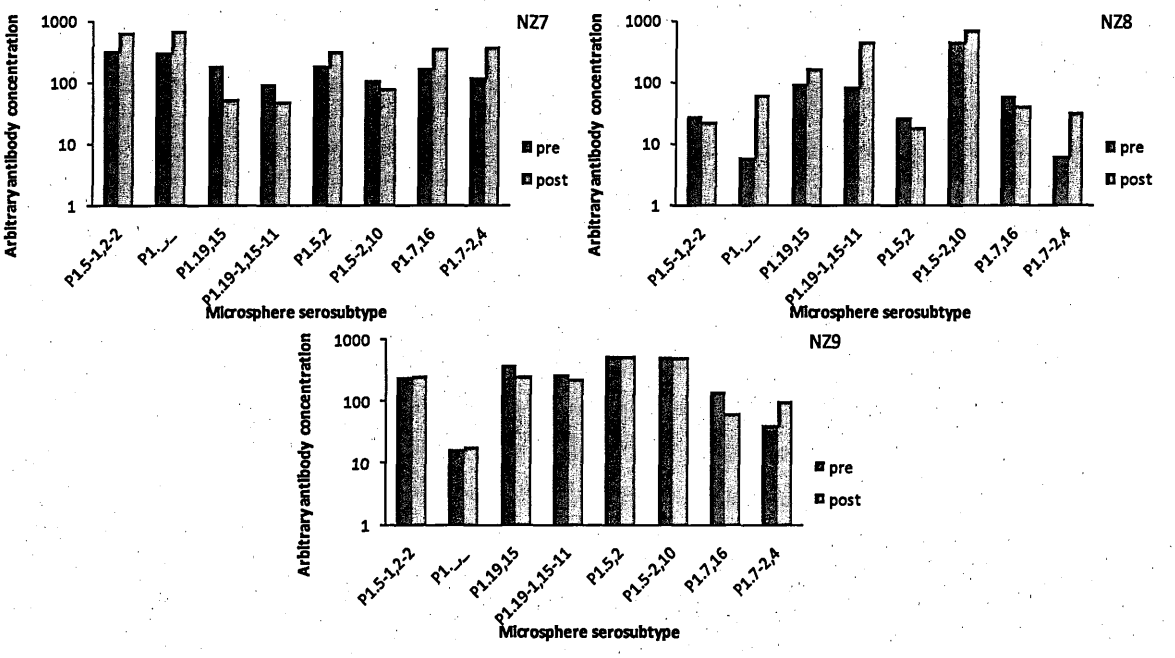


Figure 5.9 Arbitrary antibody titres from three individuals showing increased IgG titres determined against P1.7-2,4 labelled microspheres following immunisation with the P1.7-2,4 OMV vaccine. Pre-vaccination sera were taken prior to administration of the first dose, post-vaccination (boost) sera were taken six weeks following the third dose of the P1.7-2,4 vaccine. Each sample was run in duplicate at two dilutions in order to ensure antibody concentrations were within the linear range of the assay. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

As observed with the P1.7,16 OMV vaccine, sera samples were collected from laboratory workers, and as such are likely to have had previous natural exposure to meningococci isolates expressing homologous or heterologous PorA serosubtypes to those used in the Liquichip assay. The effect of vaccination on pre-existing anti-PorA antibodies must be considered. Use of this vaccine has previously been reported to induce significant increases in IgG responses to homologous and heterologous PorA antigens[342] and may explain the cross-reactive effect of the vaccine observed.

Sera from three individuals were found to have increased IgG titres determined against PorA labelled microspheres homologous to the vaccine strain, in addition to other serosubtypes (Figure 5.9). However, unlike individuals NZ2, NZ4 and NZ10, they were not found to have increased titres against to all the PorA panel serosubtypes. Individual NZ7 was found to have increased IgG titres determined against four of the eight PorA labelled microspheres, including P1.5,2; P1.5-1,2-2; P1.7,16; P1.7-2,4 and the PorA P1._,_ mutant. The largest increase in IgG titre however was seen against microspheres labelled with purified P1.7-2,4 protein with a 3.15 fold increase in IgG titre. Decreases in IgG titre were determined against the P1.19,15; P1.19-1,15-11 and P1.5-2,10 labelled microspheres. Increased titres were determined against five of the eight PorA labelled microsphere sets for individual NZ8 with the largest increase (10.26 fold) in IgG titre determined against the PorA P1._,_ mutant labelled microspheres. Increases of 5.59 and 5.24 fold were observed in IgG titre determined against the P1.19-1,15-11 and the vaccine strain homologous P1.7-2,4 labelled microspheres. Individual NZ9 was found to have an increased IgG titre specific to the vaccine serosubtype with a 2.39 fold increase in IgG titre determined against the P1.7-2,4 labelled microspheres. A decreased IgG titre was determined against the P1.7,16 serosubtype labelled microspheres. No differences were observed using any of the remaining panel PorA serosubtypes for this individual.

As for the previous samples, much of the immune response observed following vaccination with the P1.7-2,4 OMV vaccine can be explained by the presence of cross-reacting, pre-existing antibodies. High IgG concentrations determined in pre-vaccination sera complicate assessment of the immune response induced by administration of vaccine. As with the P1.7,16 OMV vaccine, the P1.7-2,4 vaccine has been shown to elicit significant increases in IgG binding to homologous and heterologous OMV antigens[342].

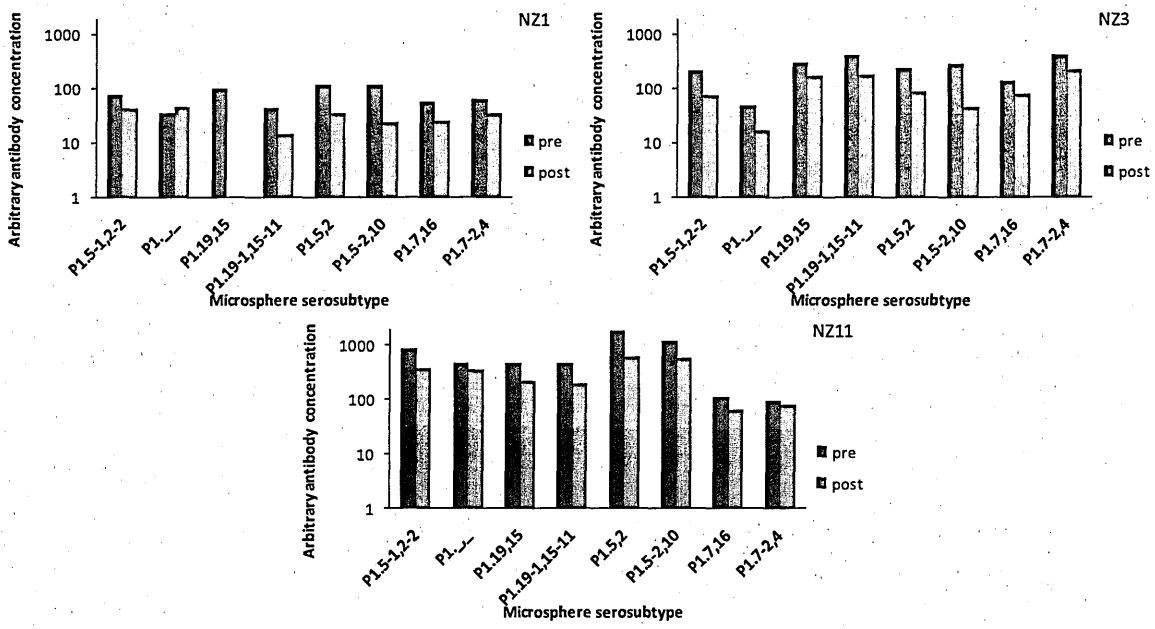


Figure 5.10 Arbitrary antibody titres from individuals found to be non-responders using the Liquichip assay with decreased IgG titres observed following immunisation with the P1.7-2,4 OMV vaccine. Pre-vaccination sera were taken prior to administration of the first dose, post-vaccination (boost) sera were taken 4 weeks following the third dose of the P1.7-2,4 OMV vaccine. Each sample was run in duplicate at two dilutions in order to ensure antibody concentrations were within the linear range of the assay. Individuals are grouped into differing magnitudes of IgG titres, shown as A and B. The post-vaccination IgG titre of NZ1, was not determined against the P1.19,15 labelled microspheres. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers following immunisation with the P1.7,16 or the P1.7-2,4 OMV vaccine.

Individuals NZ1, NZ3 and NZ11 were not determined to be responders to the P1.7-2,4 OMV vaccine when sera was analysed using the Liquichip assay. Post-vaccination sera from these individuals were found to have unchanged or decreased IgG titres determined against all PorA labelled microspheres (Figure 5.10). For these individuals, the IgG titres determined against the P1.7-2,4 labelled microspheres, homologous to the vaccine strain, were found to be unaffected with less than a 50 % decrease in IgG titre observed. The post-vaccination IgG titre determined using P1.19,15 labelled microspheres was found to be lower than the limit of detection for Individual NZ1. Decreased IgG titres were determined using microspheres labelled with P1.19-1,15-11; P1.5,2; P1.5-2,10 and P1.7-2,4 PorA purified proteins while IgG titres determined against

the remaining panel of PorA serosubtypes were unaffected following vaccination. Individual NZ3 was found to have decreased IgG titres determined using five of the eight PorA labelled microsphere panel with the largest decrease determined using the P1.5-2,10 serosubtype. Decreases were also observed using microspheres labelled with P1.5-1,2-2; P1.19-1,15-11; P1.5,2 and the P1._,_ PorA mutant purified proteins. Decreases were determined using five of the eight serosubtype labelled microspheres in sera from individual NZ11, with the largest decrease determined using the P1.5,2 labelled microspheres. Natural exposure to meningococcal isolates may result in the high titres of anti-PorA antibodies observed in pre-vaccination serum. The immune response produced following administration of the vaccine, would therefore be masked, and result in the vaccine appearing to have little or no effect on the individual.

5.5 Discussion

Binding antibody assays are traditionally used for the detection of functional and non-functional antibodies. The PorA multiplex assay is one such assay, and was successfully used for the detection of increases in serum IgG titres against specific PorA serosubtypes following vaccination with one of four different OMV vaccines in preclinical and clinical trial sera.

Differences in the effects of vaccination were more easily defined with the use of mouse sera and healthy toddler and child sera. Both these sets of sera were used to demonstrate low levels of antibodies in pre-vaccination and unvaccinated control sera alongside a specific vaccine induced increase in antibody titres in post-vaccination sera. The serosubtypes or related variants included in the current assay are also present in the Hexamen and Nonamen vaccines and as such, an increased titre against all serosubtypes was predicted[316]. Similar increases in GMTs were detected using each of the PorA labelled microspheres in murine and human toddler/child sera following vaccination with up to three doses of either the HexaMen (6-valent) or the NonaMen (9-valent) OMV vaccine in comparison to the unvaccinated control group. The antibody response detected following the booster dose of the vaccine was shown to have the largest GMTs.

The absence of pre-existing antibodies in murine serum, was confirmed by the lack of antibodies detected using the Liquichip assay in sera from unvaccinated mice. Mice do not carry meningococci and as such, murine sera would not be expected to contain pre-existing anti-meningococcal antibodies. Conversely, murine sera collected post-vaccination would only be expected to contain antibodies that recognise the epitopes of PorA serosubtypes expressed in the vaccine and was confirmed with an antibody titre measured against all PorA serosubtypes included in the assay following vaccination.

A similar response would be expected in the detection of vaccine induced responses in sera from human child sera. As a result of the low carriage rates observed in toddlers and young children, previous carriage of meningococcal strains is unlikely[43]. With serum IgG antibody levels remaining low until adolescence[114] the only anti-meningococcal antibodies present in toddler sera would be specific to the vaccine serosubtypes. Sera from unvaccinated children are therefore unlikely to contain serum antibodies to any PorA serosubtypes and the absence of pre-existing antibodies or the presence at very low levels would have been expected. This was confirmed by a significantly higher GMT detected in child/toddler serum following immunisation with the Hexamen vaccine than detected in the pre-vaccination serum sample. The high variability of individual responses was demonstrated in the post-primary vaccination samples, with high titers of specific anti-PorA antibodies detected in one sample, no response was observed in the IgG titre of the second sample. However, without the availability of pre-vaccination serum sample from the same individuals, the effect of vaccination on serum antibodies cannot be determined. It is possible that the increased level of specific PorA antibodies maybe due to the effect of vaccination boosting an existing immune response whilst antibodies to the remaining serosubtypes are not elevated until the third dose of the vaccine. This suggests that fold-differences in titre following vaccination may be just as important as absolute IgG titres in the evaluation of post vaccination responses, particularly in individuals likely to have pre-existing immunity.

Responses to vaccination were found to vary between individual human adults following vaccination with either the P1.7,16 or the P1.7-2,4 OMV vaccine. Increased IgG titres against specific PorA serosubtypes were measured in human adult sera following vaccination with three doses either of the P1.7,16 or the P1.7-2,4 OMV vaccine. Several individuals exhibited large-fold increases in antibody titre against heterologous serosubtypes as well as to the corresponding PorA serosubtype protein. Conversely, other individuals did not respond well against any of the PorA serosubtypes. It is therefore likely that a good response may be dependent on the individual rather than on the vaccine in question, highlighting the need for paired sample analysis and comparison of IgG titres both prior to and post vaccination. Due to peak carriage rates in young adults, the likelihood of carriage or previous carriage of meningococci with a homologous or heterologous serosubtype of PorA to those expressed in the vaccine is increased in adult humans[43]. With the presence of pre-existing, cross reacting antibodies, all of which have an influence on the immune response initiated in response to either a new infection or vaccination, it is difficult to distinguish between a pre-existing antibody response and a vaccine-induced response. As such the immune response elicited following vaccination of human adults may result in what appears to be non-specific responses.

Carriage itself can act as a priming event[59] with subsequent vaccination acting as a booster to elicit a specific immune response. In particular, previous carriage of meningococci with the homologous PorA serosubtype as the vaccine strain would lead to a high anti-PorA IgG titre measured against that serosubtype. Carriage of meningococci with a heterologous PorA serosubtype would initially result in a less marked immune response, increasing over time[267]. The effect of immunological priming is highlighted using sera from adults vaccinated with either the P1.7,16 or the P1.7-4,2 OMV vaccine. As the vaccines were administered to adult laboratory workers, previous carriage of meningococcal isolates and immunologic priming was probable and likely to have an effect on post-vaccination IgG titres. HexaMen has previously been shown to induce a cross-reactive antibody response against isolates with one VR identical to the vaccine serosubtype and to minor VR variants of serosubtypes included in the HexaMen vaccine[330]. The

amount of time elapsed between meningococcal carriage and vaccination would also be expected to be a major factor in the antibody response observed following administration of the vaccine. High levels of protective antibody have been seen following carriage of meningococcal strains for several months after the carriage strain has been lost[249].

The immunodominant VR of each serosubtype is of importance for vaccine design with individual serosubtype combinations having differing immunodominant VRs. Although the P1.7-2 and P1.7 epitopes are related, the P1.4 epitope is thought to be the major target for immune response for the P1.7-2,4 OMV vaccine[190] with the majority of antibodies targeted against the VR2 region of this protein. Similarly, the VR2 of P1.5-2,10 and possibly the P1.19,15 were found to be immunodominant in the Hexamen vaccine. There is some debate as to whether the VR1 or the VR2 of the P1.7,16 is the immunodominant epitope. Whilst some studies have found VR1 to be the immunodominant VR[324,91], others have determined VR2 to be the immunodominant epitope[341,349]. Whilst the variable nature of the VR regions is due to immune selection by functional antibodies in human, the production of anti-PorA antibodies not specific to either VR1 or VR2 has been reported previously[324] and would suggest an immunogenicity of the rest of the PorA structure in adults. Additionally, as previously observed, high antibody titres against the P1._,_ PorA protein may to appear elevated due to the low level of anti-P1._,_ antibodies in the standard used to quantify the response.

This assay has been successfully used in this study to analyse sera from humans, and mice vaccinated with the multivalent Hexamen and Nonamen OMV vaccines as well as the strain-specific Norwegian P1.7,16 and New Zealand P1.7-2,4 OMV vaccines. Whilst increased antibody titres were detected in sera following vaccination with each of the OMV vaccines, the detection of specific anti-PorA antibodies was found to be dependent on the source of serum. The small number of samples analysed in this chapter, limit the conclusions that can be drawn on vaccine efficacy, and would need to be repeated on a larger scale with paired pre- and post-vaccination sera samples. This assay can be further expanded for the detailed assessment of immune responses to both combination vaccines and antigenic variants.

Chapter 6. Determination of natural immunity in serum samples using both longitudinal and cross-sectional studies

6.1 Introduction

The only known reservoir of *N. meningitidis* is the human nasopharynx and colonisation by the non-invasive meningococci, resulting in asymptomatic carriage as opposed to disease, is essential for meningococcal survival and the first step of pathogenesis[46,356]. Carriage is itself an immunising event[114] leading to the development of bactericidal antibodies, directed to the colonising strain of meningococci[109]. A peak in SBA and anti-OMV IgG titres has previously been found to coincide with the peak in meningococcal carriage with evidence of the induction of SBAs as a result of carriage. The level of bactericidal antibodies may remain elevated for several months following an episode of carriage[298]. Whilst infection with *N. meningitidis* rarely leads to invasive meningococcal disease, asymptomatic carriage of meningococcal strains is a common occurrence with approximately 5 - 10 % of healthy individuals carrying *N. meningitidis* at any time[303]. Increased rates of carriage, approximately 25 %[302] were found in adolescents and young adults. In particular, increased rates of meningococcal carriage have been found in military recruits and college students as a consequence of closed, crowded living conditions and mixing with new communities[287,36]. Mobilisation of current strains is the principal method of transmission of pathogenic and commensal strains of meningococci, and infection is usually from within the healthy population rather than from individuals with the disease[36]. As such, an understanding of transmission of meningococcal isolates is an important part of public health management.

Carriage studies provide useful information about the epidemiology and pathogenesis of meningococcal disease. Risk factors for carriage in young adults, such as smoking, kissing and attending night clubs have been identified through carriage studies[43,46,141], in additional to providing knowledge that can be used for widespread outbreak management[356].

This chapter uses the Liquichip assay to analyse sera samples collected as part of two separate studies. Serum samples were collected from healthy students from the University of Nottingham as part of a carriage study carried out jointly by the University of Leicester and the University of Nottingham, to analyse phase variable genes of meningococcal isolates using genetic and serological methods. Throat swabs, blood and saliva samples were taken from 190 first year students, recruited from five halls of residence. Repeat samples were taken from the same individuals at t = 4, t = 12 and t = 24 weeks. Isolates were identified as meningococci by the presence of *crgA*, *ctrA*, and *porA* genes. DNA analysis was used for serogrouping, *porA* typing, *fetA* typing, MLST and repeat tract analysis[25]. A selection of these samples were used in this study, and individuals were grouped into categories consisting of: eight non-carriers, of whom four individuals provided several samples over the period of 4 - 24 weeks, where no serosubtype was identified; five new acquirers, where serosubtype was detected following a non-carrier sample (Table 6.2); ten persistent carriers, where the same serosubtype was detected over multiple time points (Table 6.4); and eleven eliminators, where a loss of serosubtype was observed following carriage (Table 6.6). Using the Liquichip assay, arbitrary antibody concentrations were calculated and differences in titres over a 24 week period were analysed to ascertain whether antibody concentration had a direct effect on carriage of the colonising meningococci and the effect of strain acquisition.

Sera from five hundred healthy individuals ranging from 15 – 19 years of age were obtained from the HPA SEU[7] collection[215,300]. Samples were randomly chosen with approximately 100 representative samples taken from those collected in 1990, 1994, 1998, 2002 and 2006. This collection is comprised of residual samples submitted by participating laboratories, based around England and Wales, following routine diagnostic testing representing the entire age range of the population. Whilst the reason the blood sample was taken is not recorded, samples from immunocompromised individuals are excluded. Prior to 1996, samples submitted for testing for antibodies to HIV or Hepatitis B infection were also excluded, however these are now included in

[7] http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/SeroepidemiologyProgramme/

order to provide a complete sample. Each sample is assigned a unique identity number and the age (or date of birth) and sex of the donor, year of collection, and participating laboratory are recorded, whilst all laboratory numbers are removed ensuring samples are anonymous. Sera samples from this collection were intended to examine potential changes in exposure to different meningococci over time, and have been tested for a variety of diseases including Measles, Mumps and Rubella, Diphtheria and Tetanus[215].

6.2 Aims of this chapter

The aims of this chapter were:

- To examine the effect of meningococcal carriage on host immunity by measurement of IgG concentrations in sera collected from university students with known carriage states over a 24 week period as part of a longitudinal study.
- To examine the IgG titre of antibodies specific to serosubtypes of PorA in sera representative of young adults throughout England and Wales over a 16 year period (1990 – 2006).

6.3 Results

6.3.1 Association of increases in IgG concentrations in direct comparison to carriage status

6.3.1.1 Non-carriers

Non-carrier serum was found to exhibit low level IgG responses to all of the PorA serosubtypes in this assay (Figure 6.1). Whilst direct comparison cannot be made between IgG titres directed against each of the PorA serosubtypes, the GMT of IgG against all of the PorA subtypes were significantly higher than for the PorA mutant P1._,_, in which the two major antigenic variable regions (VR1 and VR2) had been deleted ($p \leq 0.05$).

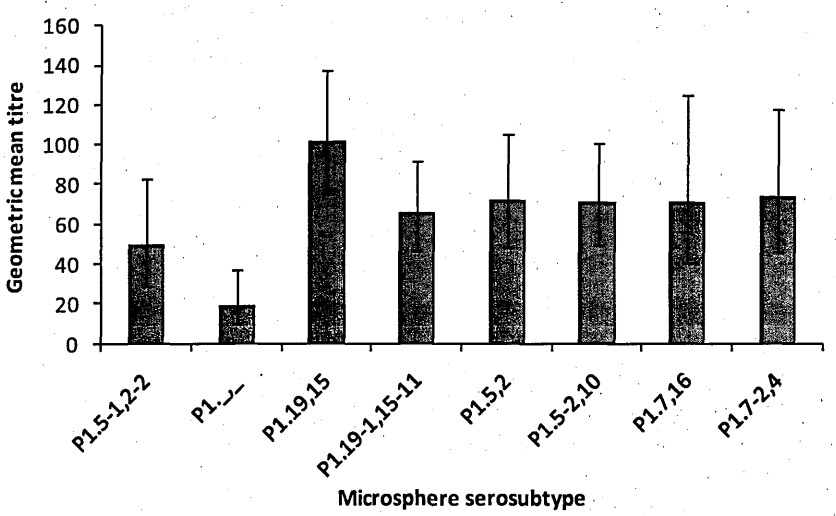


Figure 6.1 Geometric mean IgG titres of sera from healthy university students from the University of Nottingham (error bars represent 95 % confidence intervals). Responses are shown as the GMT of twelve samples collected from eight individuals (multiple samples were used for four individuals). Serum was assayed using two replicate samples at two dilutions in order to ensure the antibody concentration was within the linear range of the assay. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers immunised with three doses of the P1.7,16 or the P1.7-2,4 OMV vaccine as described previously.

Multiple sera samples from four individuals were collected at multiple time points. The initial sample was collected at t = 0 weeks, and additional samples were collected at t = 4, t = 12 and t = 24 weeks. Samples for every time point were not available for each of these four individuals. Little difference was observed in IgG responses in sera from the same individual sampled at multiple time points, with a less than 1.3 fold increase observed for all serosubtypes (Table 6.1). As a result of the low IgG titres of anti-PorA measured in these sera, differences in the calculated IgG titre determined against each of the PorA labelled microsphere sets in these samples are likely to be a result of noise, or fluctuations in the system, with a difference or one or two MFI, giving a large difference in assigned arbitrary concentration.

Table 6.1 Fold increase in antibody concentrations observed between samples from the same individual taken at timed intervals between t = 0 and t = 4 - 24 weeks. The average fold difference is shown with the 95 % confidence interval (CI) in brackets.

Sample	Protein labelled microspheres							
	P1.5-1,2-2	**P1._,_**	**P1.19,15**	**P1.19-1,15-11**	**P1.5,2**	**P1.5-2,10**	**P1.7,16**	**P1.7-2,4**
Individual A	0.970	1.040	0.927	0.810	1.000	0.946	0.957	0.941
Individual B	1.010	1.210	1.250	1.195	1.233	0.930	1.124	1.023
Individual C	1.264	1.286	1.477	1.539	1.135	1.125	1.635	1.408
Individual D	0.755	0.838	0.718	0.684	0.826	0.850	0.480	0.612
Average (C.I)	**1.000 (0.33)**	**1.094 (0.32)**	**1.093 (0.54)**	**1.057 (0.62)**	**1.049 (0.28)**	**0.963 (0.18)**	**1.049 (0.76)**	**0.996 (0.52)**

6.3.1.2 New Carriers

To investigate whether the recent acquisition of the meningococcus stimulates host immunity resulting in an increase in circulating IgG, sera from five individuals (Table 6.2), who had acquired meningococcal carriage over the course of the study, were analysed using the Liquichip assay. Samples from the same individual were analysed, with samples collected at various intervals to monitor the induction of PorA variant specific antibodies following acquisition of carriage. Of the five individuals known to have acquired carriage of meningococci, each was found to have been colonised with isolates expressing different serosubtypes of PorA (Table 6.2). These were: P1.21,16; P1.21-7,16; P1.5-1,2-2; P1.5,2; and P1.5-1,10-1. Whilst all of these serosubtypes are not included in the Liquichip assay, closely related minor variants were present and were used to analyse sera responses.

Table 6.2 Individuals found to have acquired carriage of meningococci during this study. The corresponding microsphere PorA type is given, with the homologous serosubtype (or minor variant of) shown in bold.

Individual	Serosubtype of Acquired Isolate		Microsphere PorA type	
	VR1	VR2	VR1	VR2
N1	21	**16**	7	**16**
N2	21-7	**16**	7	**16**
N3	**5**	**2**	**5**	**2**
N4	**5-1**	**10-1**	**5-2**	**10**
N5	**5-1**	**2-2**	**5-1**	**2-2**

Acquisition of carriage was associated with an increase in IgG response to the specific PorA serosubtype of the carried isolate. Figure 6.2 shows IgG responses to each of the PorA serosubtypes included in the assay. The effect of acquisition on heterologous serosubtypes was found to vary among individuals. Individuals N1 and N2 were found to exhibit increased IgG concentrations in response to one or more of the heterologous serosubtypes of PorA. Responses to the remaining serosubtypes were largely unaffected for individuals N3 and N4, whilst increases

in IgG concentration were observed in response to all serosubtypes for individual N5 as shown in Figure 6.2. As observed with the non-carrier individuals, the lowest IgG concentration was determined against the mutant P1._,_ PorA serosubtype for all individuals.

Individual N1, identified as a new carrier of a meningococcal isolate with the P1.21,16 serosubtype of PorA, was shown to exhibit a 2.1 fold increase (Table 6.3) in IgG concentration directed towards the corresponding P1.7,16 labelled microspheres. A 13.5 fold increase was also observed in antibody concentration directed against the P1.5-2,10 serosubtype, a 3.7 fold increase against the P1.19-1,15-11 serosubtype and a 1.8 fold increase against the P1.5-1,2-2 serosubtype of PorA. Similarly, a 19.3 fold increase in IgG concentration directed towards the P1.7,16 serosubtype labelled microspheres was observed for N2, following acquisition of a carriage isolate with the P1.21-7,16 serosubtype PorA. Increased IgG concentrations, determined against the P1.19-1,15-11 and P1.5-2,10 PorA labelled microspheres, were observed with responses to all remaining serosubtypes unaffected. These data appear to suggest cross-reactivity between PorA epitopes, in particular between antibodies directed against the P1.21,16 serosubtype of PorA, and both the P1.5-2, 10 and P1.19-1,15-11 protein labelled microsphere. Interestingly, cross-reactivity is not observed with the related P1.19,15 protein labelled microspheres. These proteins share the same variant of VR3 and it is possible that serum antibodies raised in response to meningococcal isolates with the P.21,16 serosubtype of PorA are binding to this epitope on the purified PorA proteins.

Individuals N3 and N4 exhibited large increases in sera IgG responses, specific to the serosubtype of the carriage isolate acquired. Following colonisation of individual N3 with a carriage isolate expressing the P1.5,2 serosubtype of PorA, increased IgG concentrations were observed against both the P1.5,2 and P1.5-1,2-2 serosubtype labelled microspheres, with a 7.7 fold and a 3.9 fold increase observed, respectively, up to 12 weeks following colonisation. This increase was amplified in the final sera sample, taken up to 24 weeks post-colonisation with a 65.6 fold and 34.5 fold increases, respectively. Subsequent colonisation with a P1.5-1,10-1 carrier isolate was identified at the University of Nottingham. Whilst an increase in IgG concentration directed

against the P1.5-1,2-2 labelled microspheres was observed, no increase was observed using the P1.5-2,10 labelled microspheres (Table 6.3). With increases in serosubtype specific antibody titre have been reported as early as two weeks following the onset of carriage, it is likely that colonisation with the P1.5-1,2-2 carrier isolate was very recent.

Acquisition of a meningococci isolate expressing the P1.5-1,10-1 PorA by Individual N4, resulted in a 10.8 fold increase in IgG concentration determined using the corresponding P1.5-2,10 serosubtype labelled microspheres. A more limited, 2.4 fold increase in IgG concentration was determined using the P1.5-1,2-2 labelled microspheres, along with a 2.6 fold increase against the related P1.5,2 labelled microspheres (Table 6.3). This suggests the immunodominance of the VR2 region of the PorA protein as a target for antibody binding.

Individual N5, a new carrier of a carriage isolate expressing the P1.5-1,2-2 serosubtype of PorA, exhibited an increase in IgG concentration in response to all serosubtypes of PorA included in this assay. The largest increase was directed against the P1.19,15 and P1.19-1,15-11 labelled microspheres with 22.3 and 22.9 fold increases observed, respectively. Similar increases in IgG concentrations were observed for related serosubtypes. An increase of 11.8 and 13.1 was determined using the P1.5-1,2-2 and P1.5,2 labelled microspheres, respectively. A 3.0 and 3.6 fold increase observed using microspheres labelled with the P1.7,16 and P1.7-2,4 serosubtypes of PorA, respectively (Table 6.3 and Figure 6.2). An increase in IgG concentration directed to all serosubtypes of PorA may have been a result of previous carriage and clearance of meningococcal isolates with a serosubtype homologous to the protein labelled microspheres, and boosting of an existing immune response following an episode of carriage.

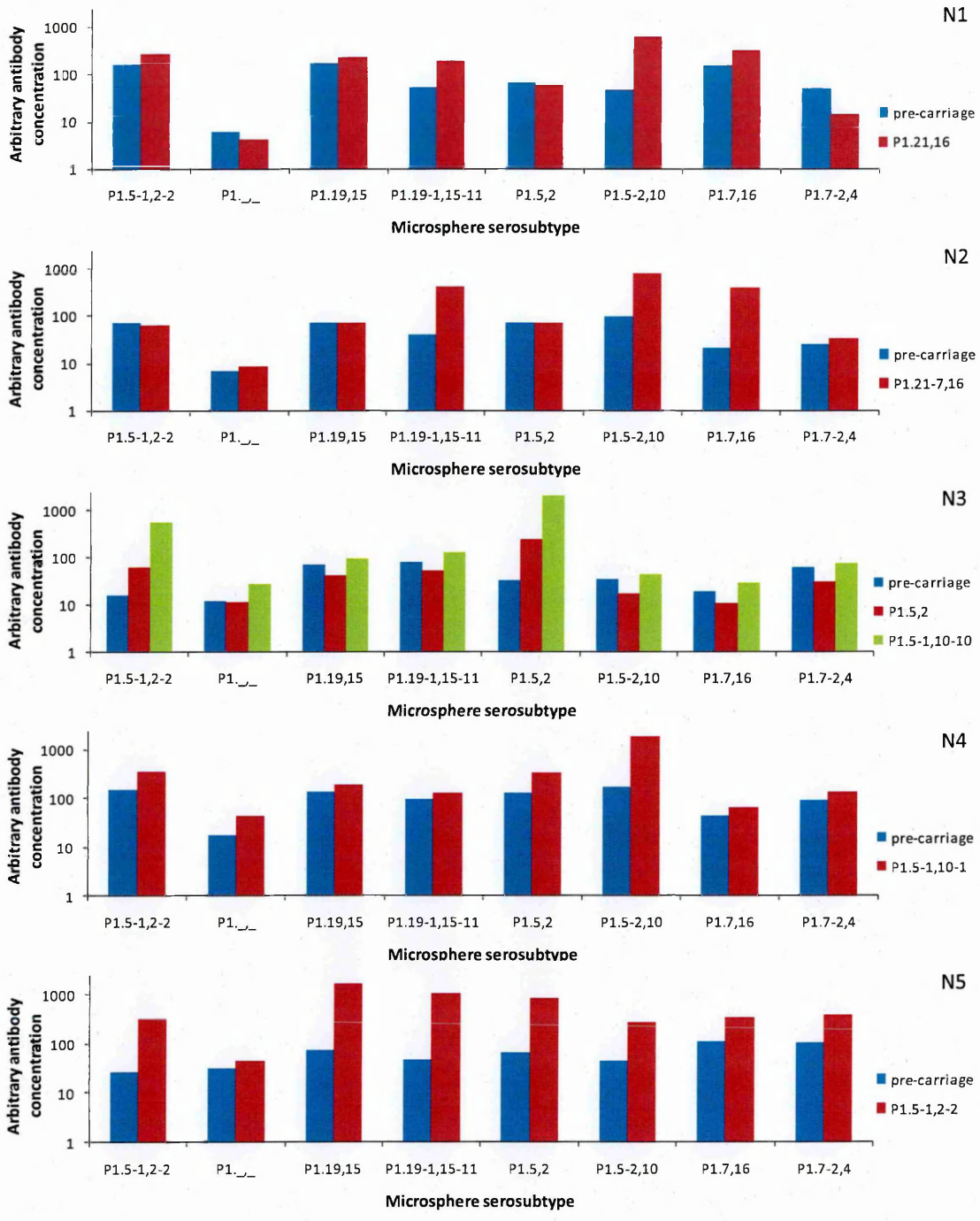


Figure 6.2 IgG titres of sera from individuals known to have acquired meningococcal carriage. Individuals were found to be non-carrier of meningococci at initial sampling, however were found to have acquired meningococcal carriage at a later time point. Individuals N2 and N3 were found to have acquired carriage of the meningococci at 12 weeks. The remaining individuals were found to have acquired carriage of meningococci at 24 weeks. Whilst the homologous serosubtype was not tested for in every case, closely related variants are present, and IgG responses are expected to be directed to these variants. All samples were tested in duplicate at two dilutions of serum. Titres were determined using a standard curve composed of pooled sera from laboratory workers following immunisation with the P1.7,16 or the P1.7-2,4 OMV vaccine.

Table 6.3 Fold increase in IgG concentrations observed between samples from the same individual at timed intervals following acquisition of carriage. Increases were calculated in relation to the previous timed sample where no meningococcal carriage isolate could be identified. Samples from two time periods were used for each individual with the exception of N3, where samples from three time points were available. Samples were run in duplicate at two dilutions of serum to ensure antibody concentrations were within the linear range of the assay. Numbers in bold represent the serosubtype of the acquired meningococcal isolate.

Sample	Protein labelled microspheres							
	P1.5-1,2-2	**P1._,_**	**P1.19,15**	**P1.19-1,15-11**	**P1.5,2**	**P1.5-2,10**	**P1.7,16**	**P1.7-2,4**
N1	1.8	0.7	1.4	3.7	0.9	13.5	**2.1**	0.3
N2	0.9	1.2	1.0	10.3	1.0	8.5	**19.3**	1.3
N3	3.9	1.0	0.6	0.7	**7.7**	0.5	0.6	0.5
N3b	34.5	2.3	1.4	1.6	**65.6**	1.3	1.5	1.2
N4	2.4	2.5	1.4	1.3	2.6	**10.8**	1.4	1.5
N5	**11.8**	1.4	22.3	22.9	13.1	6.2	3.0	3.6

6.3.1.3 Persistent carriers

To evaluate the effect of persistent carriage of the meningococcus on the serosubtype-specific antibody concentrations, sera from ten individuals (Table 6.4) identified as persistent carriers of meningococcal isolates over at least two sampling periods over the course of this study, were analysed on the Liquichip using the panel of PorA-coated microspheres. Samples from the same individual were analysed, with samples collected at various intervals to monitor differences in IgG concentration of PorA variant specific antibodies during persistent carriage. Ten individuals were known to have persistent carriage of meningococcal isolates with one of five serosubtypes of PorA. These were P1.21,16; P1.5-1,10-1; P1.5,2; P1.5-1,2-2 and P1.19-1,15-11. Whilst these serosubtypes are not all included in the Liquichip assay, closely related variants were present and were used to analyse sera responses (Table 6.4).

Table 6.4 Individuals found to be persistent carriers of a meningococcal isolate with a single serosubtype of PorA. The corresponding microsphere PorA serosubtype is given, with the homologous serosubtype (or minor variants of) shown in bold.

Individual	Serosubtype of Carriage Isolate		Microsphere PorA type	
	VR1	VR2	VR1	VR2
P1	21	**16**	7	**16**
P2	21	**16**	7	**16**
P3	21	**16**	7	**16**
P4	21	**16**	7	**16**
P5	**5-1**	**10-1**	**5-1**	**10**
P6	**5-1**	**10-1**	**5-1**	**10**
P7	**5**	**2**	**5**	**2**
P8	**5**	**2**	**5**	**2**
P9	**5-1**	**2-2**	**5-1**	**2-2**
P10	**19-1**	**15-11**	**19-1**	**15-11**

IgG responses to the panel of PorA coated microsphere, over a 12 – 24 week period are shown in Figure 6.3, Figure 6.4 and Figure 6.5. A decrease in IgG concentration was determined using

microspheres labelled with the PorA serosubtype homologous to the carriage isolate for many serotypes with the exception of P1.5-1,10-1. Individuals P5 and P6 exhibited 1.43 and 1.13 fold increases in IgG concentrations directed to the P1.5-2,10 PorA labelled microspheres, respectively (Table 6.5). The effect of persistent carriage on the IgG concentration to the remaining non-homologous panel PorA-serosubtypes was found to vary by individual. IgG titres determined using microspheres labelled with non-homologous PorA serosubtypes were largely unaffected for Individuals P3, P4, P5, P6 and P8 (Figure 6.4 and Figure 6.5), and more pronounced for others such as Individual P1 (Figure 6.3). Changes in IgG concentration were not found to be serosubtype-specific following periods of persistent carriage for any of the Individuals tested in this study. Duplicate samples for sera collected from individuals were assayed using the liquichip assay. As no statistical analysis was performed on these data, these comments are merely observations of trends.

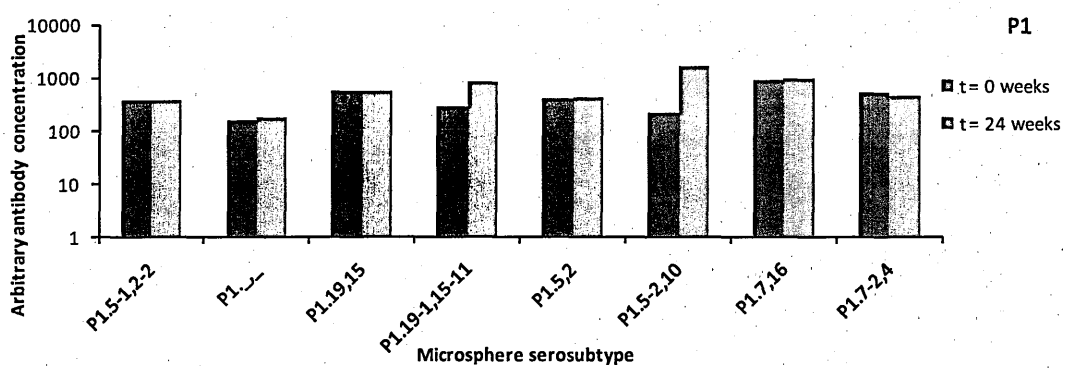


Figure 6.3 IgG titres of sera from individual P1, known to be a persistent carrier of a meningococcal isolate with the P1.21,16 serosubtype of PorA. Sera responses to specific serosubtypes were determined following analysis with the Liquichip assay. Sera were tested in duplicate at two dilutions, to ensure the antibody concentration was within the linear range of the assay. Titres were determined using a standard curve composed of pooled sera from laboratory workers following immunisation with the P1.7,16 or the P1.7-2,4 OMV vaccine.

A known carrier of the P1.21,16 serosubtype, individual P1 was found to have increased concentrations of IgG directed against the P1.19-1,15-11 and the P1.5-2,10 PorA labelled microspheres (Figure 6.3), with an increase of 2.82 and 7.37 observed, respectively (Table 6.5).

Responses to the remaining panel of PorA labelled microspheres, including the P1.7,16 serosubtype, were unaffected with a concentration difference ranging from a minimum of 0.89 to a maximum of 1.14 across the panel of PorA labelled microspheres. As for new carriers, cross reactivity, due the shared VR3 region between the antibodies directed against the P1.21,16 serosubtype of PorA, and both the P1.5-2, 10, and P1.19-1,15-11 protein labelled microspheres is suggested.

During persistent carriage of a meningococcal isolate with the P1.21,16 serosubtype of PorA, individual P2 was found to have decreased IgG concentrations in response to every serosubtype of PorA used in this assay over a 24 week period (Figure 6.4). The magnitude of decrease varied with a minimum 0.85 fold difference observed using the P1.5-1,2-2 labelled microspheres, similar differences were observed against the P1.19,15; P1.5,2; and the P1.7,16 serosubtypes. The maximum decrease in IgG concentration was observed against the P1._,_ labelled microspheres with a 0.15 fold difference over the 24 week study period. The effect of persistent carriage of a P1.21,16 serosubtype meningococcal isolate by individual P3, was found to be serosubtype specific, with increases in IgG concentration determined in response to the P1.5,2 and the P1.5-2,10 serosubtype labelled microspheres, with 1.44 and 1.23 fold differences, respectively (Figure 6.4). Decreases in IgG concentration were observed in response to all other serosubtypes on the PorA panel. The maximum decrease was determined against the P1.19-1,15-11 and P1.19,15 labelled microspheres with a 0.61 fold and a 0.74 fold difference observed, respectively over a 24 week period. GMTs determined using the panel of PorA labelled microspheres were largely unaffected for individual P4, also a carrier of a P1.21,16 serosubtype carrier isolate (Figure 6.4). Increased IgG concentrations were found in response to P1._,_ and P1.7,16 labelled microspheres with a 1.91 and 1.38 fold difference, respectively, whilst differences in the IgG concentration in response to the remaining panel of PorA labelled serosubtypes ranged from a minimum 0.82 to a maximum 1.14 fold difference. Differences in IgG concentration in response to the P1._,_ labelled microspheres may have been exaggerated, due to the low IgG concentrations in response to the P1._,_ PorA mutant. Decreases in sera responses were, again, observed in response to all

serosubtypes of PorA used in this assay for individual P10 during persistent carriage of a meningococcal isolate with a P1.19-1,15-11 over a 24 week period (Figure 6.4). The smallest difference in IgG concentration was found to be directed towards the homologous P1.19-1,15-11 PorA labelled microspheres with minimum 0.89 difference. The maximum decrease was determined in response to the P1.7-2,4 serosubtype labelled microspheres with a concentration difference of 0.24 observed over a 24 week period.

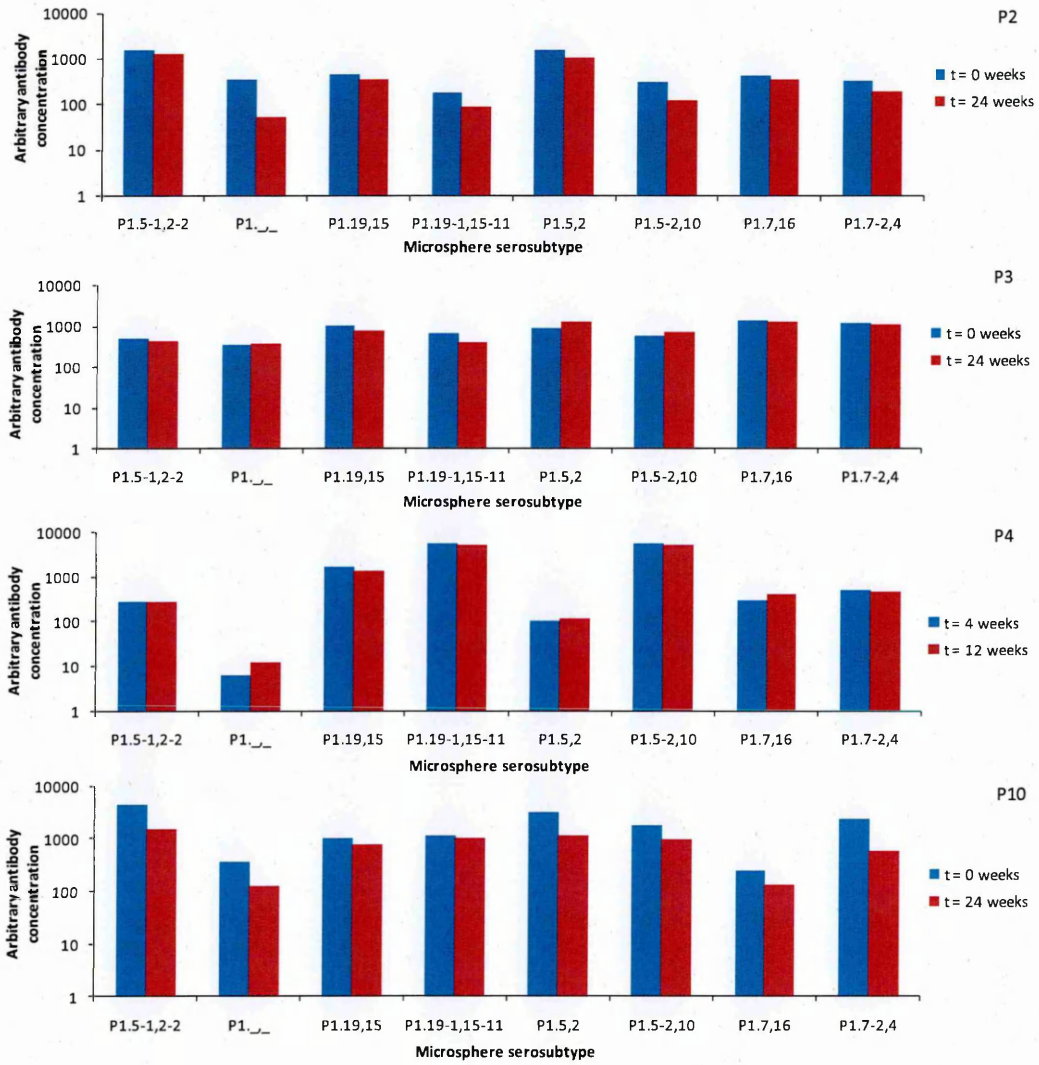


Figure 6.4 IgG titres of sera from individuals known to be persistent carriers of a meningococcal isolate. Sera responses to specific serosubtypes were determined following analysis with the Liquichip assay. Sera were tested in duplicate at two dilutions. Titres were determined using a standard curve composed of pooled human sera from healthy laboratory workers vaccinated with either the P1.7,16 or the P1.7-2,4 OMV vaccine. Individuals P2 – P4 were found to be carriers of a meningococcal isolate with the P1.21,16 serosubtype of PorA. Individual P10 was found to be a carrier of a meningococcal isolate with the P1.19-1,15-11 serosubtype.

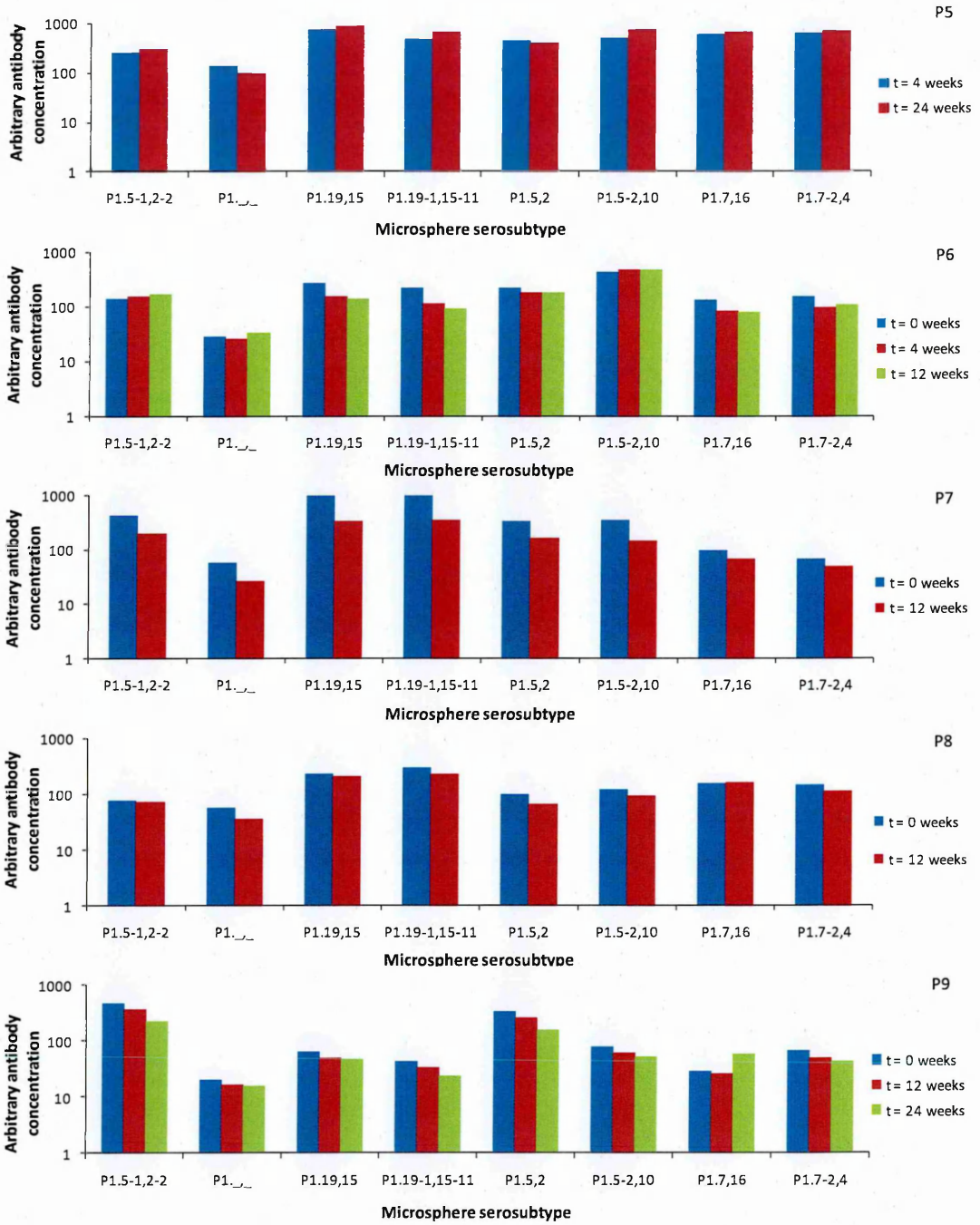


Figure 6.5 IgG titres of sera from individuals known to be persistent carriers of a meningococcal isolate. Sera responses to specific serosubtypes were determined following analysis with the Liquichip assay, using a standard curve composed of pooled human sera from individuals vaccinated with either the P1.7,16 or the P1.7-2,4 OMV vaccine. Two samples were analysed for all individuals with the exception of individuals P6 and P9 where samples from three time points were available. Sera were tested in duplicate at two dilutions, to ensure the antibody concentration was within the linear range of the assay. Individuals P5 and P6 were known to be persistent carriers of a meningococcal isolate with the P1.5-1,10-1 serosubtype, Individuals P7 and P8 carriers with the P1.5,2 serosubtype and Individual P9 a carrier of an isolate with the P1.5-1,2-2 serosubtype.

Persistent carriage was again found to have a limited effect on the antibody responses of individuals P5 and P6, both found to be carriers of a meningococcal isolate with the P1.5-1,10-1 serosubtype. For Individual P5, differences in IgG concentrations ranged from a minimum of 0.74 determined against the P1._,_ labelled microspheres, to a maximum 1.43 increase determined using P1.5-2,10 PorA labelled microspheres (Table 6.5). Similarly, for individual P6, differences in IgG concentrations ranged from a minimum 0.41 fold difference determined using the P1.19-1,15-11 labelled microspheres, to a maximum 1.23 fold increase determined against the P1.5-1,2-2 PorA labelled microspheres over a 12 week period. No difference in IgG concentration in response to any of the panel PorA labelled microspheres was observed in sera collected at t = 4 and t = 12 week timed intervals (Figure 6.5). Non-serosubtype specific decreases in IgG concentrations were observed in response to all of the PorA labelled microspheres for individuals P7 and P8, following persistent carriage of a meningococcal isolate with a P1.5,2 serosubtype over a 12 week period (Figure 6.5). Responses directed to six of the eight panel PorA serosubtypes were found to have decreased to less than half of the initial IgG concentration (0.38 – 0.48 fold) for individual P7. Differences determined against the remaining P1.7,16 and P1.7-2,4 serosubtype labelled microspheres were less marked, with a 0.70 and 0.72 fold difference, respectively (Table 6.5). Whilst decreased IgG concentrations were also observed for individual P8, these were not as marked as for individual P7, and ranged from a difference in IgG concentration from 0.62 – 1.05 determined against the P1._,_ PorA mutant and P1.7,16 PorA labelled serosubtypes, respectively. Decreases in serological responses to the panel of PorA labelled microspheres were observed, to an similar extent, following persistent carriage of a meningococcal isolate with a P1.5-1,2-2 serosubtype for over a 12 week period by individual P9 (Figure 6.5). Surprisingly, the difference in IgG concentration determined using P1.7,16 PorA labelled microspheres was found to change from a minimum 0.90 fold at t = 12 weeks, to a maximum 2.04 fold increase at t = 24 weeks (Table 6.5). This difference was not observed in IgG titre determined using any of the other PorA labelled microspheres.

Carriage of meningococcal isolates may be prolonged (over a few months), intermittent (last days to several weeks) or transient[35,286]. As such a carriage study performed over a period of 24 weeks can be useful for demonstrating all aspects of carriage, from acquisition of carriage, through persistent carriage, to clearance of the meningococcal isolate. In particular, a study of this length can be a useful tool in the observation of trends in meningococcal spread and carriage within a confined population such as students returning to university following a long period of absence, or students from diverse locations attending university for the first time. This is particularly true where subjects are unlikely to return over a prolonged sampling period. Carriage studies performed over a short period can only be used for the assessment of carriage prevalence within a given population.

Prolonged episodes of meningococcal carriage show the benefit of carriage studies performed over a much longer time period, often years. There are several advantages to a prolonged study, including monitoring the effects of persistent carriage, the rise and decline of bactericidal and binding antibodies raised in response to carriage of a meningococcal isolate. Studies such as these can be useful in predicting trends in the carriage of meningococcal isolates over a much larger population and geographical area. Prolonged studies are also for the evaluation of vaccines, and their effect on herd immunity.

Table 6.5 Fold increases in IgG concentrations observed between samples from the same individual known to be persistent carriers of a meningococcal isolate at timed intervals. Differences were calculated in relation to the primary sample available for each individual. Samples from two time periods were used for each individual. For P6 and P9, where samples from three time points were available, both differences are given, denoted P6b and P9b. Microsphere serosubtypes corresponding to the carriage isolate are shown in bold.

Sample	Protein labelled microspheres							
	P1.5-1,2-2	**P1._,_**	**P1.19,15**	**P1.19-1,15-11**	**P1.5,2**	**P1.5-2,10**	**P1.7,16**	**P1.7-2,4**
P1	1.03	1.14	1.02	2.82	1.07	7.37	**1.11**	0.89
P2	0.85	0.15	0.76	0.48	0.66	0.41	**0.82**	0.61
P3	0.85	1.05	0.74	0.61	1.44	1.23	**0.93**	0.91
P4	0.97	1.91	0.82	0.99	1.14	0.92	**1.38**	0.95
P5	1.14	0.74	1.23	1.40	0.87	**1.43**	1.12	1.11
P6	1.11	0.90	0.56	0.51	0.83	**1.12**	0.64	0.64
P6b	1.26	1.16	0.51	0.41	0.80	**1.13**	0.60	0.70
P7	0.48	0.46	0.34	0.36	**0.47**	0.42	0.70	0.74
P8	0.94	0.62	0.87	0.79	**0.69**	0.79	1.05	0.78
P9	**0.77**	0.81	0.78	0.77	0.78	0.77	0.90	0.72
P9b	**0.47**	0.79	0.73	0.56	0.49	0.64	2.04	0.65
P10	0.35	0.33	0.73	**0.89**	0.35	0.54	0.53	0.24

6.3.1.4 Clearance of carriage

To investigate whether increases in circulating IgG resulted in clearance of the meningococcus, sera from eleven individuals (Table 6.6), found to have cleared carriage of the meningococcal isolate over the course of the study, were analysed on the Liquichip using the panel of PorA-coated microspheres. Samples from the same individual were analysed, with samples collected at various intervals to monitor the induction of PorA variant specific antibodies preceding clearance of carriage. Twelve individuals were known to have cleared carriage of the meningococcus with one of six serosubtypes of PorA. These were P1.21,16; P1.21-7,16; P1.5-1,10-1; P1.5-1,10-26; P1.5,2 and P1.5-1,2-2. Whilst these serosubtypes are not all included in the Liquichip assay, closely related variants were present and were used to analyse serological responses.

Table 6.6 Individuals found to have cleared meningococcal carriage, over the course of this study. The corresponding microsphere PorA type is given, with the homologous serosubtype (or minor variants of) shown in bold.

Individual	Serosubtype of Cleared Isolate		Microsphere PorA type	
	VR1	VR2	VR1	VR2
C1	21	**16**	7	**16**
C2	21	**16**	7	**16**
C3	21	**16**	7	**16**
C4	21	**16**	7	**16**
C5	21-7	**16**	7	**16**
C6	**5-1**	**10-1**	**5-1**	**10**
C7	**5-1**	**10-1**	**5-1**	**10**
C8	**5-1**	**10-26**	**5-1**	**10**
C9	**5**	**2**	**5**	**2**
C10	**5**	**2**	**5**	**2**
C11	**5-1**	**2-2**	**5-1**	**2-2**

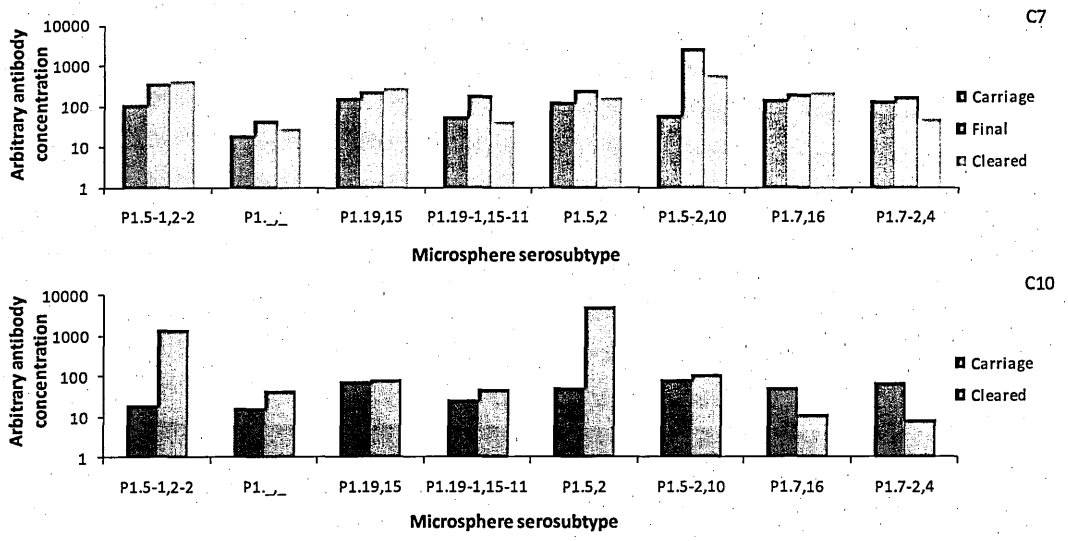


Figure 6.6 Antibody concentration for individuals known to have cleared carriage of meningococcal isolates. Sera responses to specific serosubtypes was determined, following analysis with the Liquichip assay. IgG titres were determined using a standard curve composed of pooled human sera from healthy laboratory workers vaccinated with either the P1.7,16 or the P1.7-2,4 OMV vaccine. Three samples were analysed for C7, a carrier of a meningococcal isolate with the P1-5-1,10-1 serosubtype, to determine increases in serosubtype specific IgG responses both prior to and post clearance of the meningococcal isolate. Two samples were analysed for C10, found to be a carrier of a meningococcal isolate with the P1.5,2 serosubtype, to determine increases in serosubtype specific IgG responses following clearance.

Two to three samples taken over a period of 12 - 24 weeks were analysed for each individual. Greater than 2 fold increases were found in the level of PorA specific antibodies either when the carriage isolate was last detected in the sample, or at the following time point where the carriage isolate could no longer be detected for six of the eleven individuals analysed (Table 6.6). The greatest increase in IgG titre was observed immediately preceding clearance. In particular, individuals C7 and C10 were found to have a 44.09 and 94.81 fold increase, respectively in IgG titre specific to the serosubtype of the carried isolate (Figure 6.6). Although an increase in IgG titre was not determined for Individuals C3 and C5, high concentrations of serosubtype specific antibodies homologous to the serosubtype of the carriage isolate were found (Figure 6.8). No increase was found preceding clearance of the P1.5-1,2-2 carriage isolate in C11, however, serum

was only available from one individual found to have cleared carriage of a meningococcal isolate with the P1.5-1,2-2 serosubtype, so it was not possible to determine whether this was an individual response, or a serosubtype specific response (Figure 6.9).

The largest increase in IgG concentration was observed in response to the P1.5,2 labelled microspheres, homologous to the serosubtype of the meningococcal isolate following clearance. C10 exhibited a 94.81 fold increase, with a similar 74.77 increase in PorA antibodies in response to P1.5,2 and related P1.5-1,2-2 labelled microspheres, respectively. Similarly a 44.09 fold increase was also observed for Individual C7 in response to the P1.5-2,10 labelled microspheres prior to clearance of a meningococcal isolate with the P1.5-1,10-1 serosubtype. Analysis of the C7 post-clearance sample revealed a reduced 9.92 increase in response to the same microsphere set (Figure 6.6).

Clearance of carriage was preceded by a greater than two fold increase, in serosubtype specific antibodies for individuals C1, C2, C6 and C9 (Figure 6.7). Sera responses for individuals C1 and C2, known to have cleared carriage of a meningococcal isolate with a P1.21,16 serosubtype, were found to be largely serosubtype specific, with responses to the remaining panel of PorA labelled microsphere largely unaffected. A 1.78 fold increase was determined using P1.7-2,4 labelled microspheres for C1, however this may have been due to the low IgG concentrations determined for this sample. Clearance of meningococcal isolates on individuals C6 and C9 was found to have a non-serosubtype specific effect (Figure 6.7). Increases in IgG concentration were determined against all PorA serosubtype labelled microspheres for C6, known to have cleared carriage of a meningococcal isolate with a P1.5-1,10-1 serosubtype. A minimum 1.49 fold increase was observed against P1.5,2 and P1.7,16 labelled microspheres and a maximum 2.31 fold increase observed in response to P1.19,15 labelled microspheres. Decreased IgG concentrations were observed for C9 against P1._,_ and P1.7,16 labelled serosubtypes, with a 0.60 and a 0.85 fold difference observed, respectively. Increased IgG responses were determined against the remaining panel of PorA labelled microspheres. The maximum increase in IgG titre was

determined against P1.5,2 labelled microspheres, homologous to the serosubtype of the carriage isolate (Table 6.6).

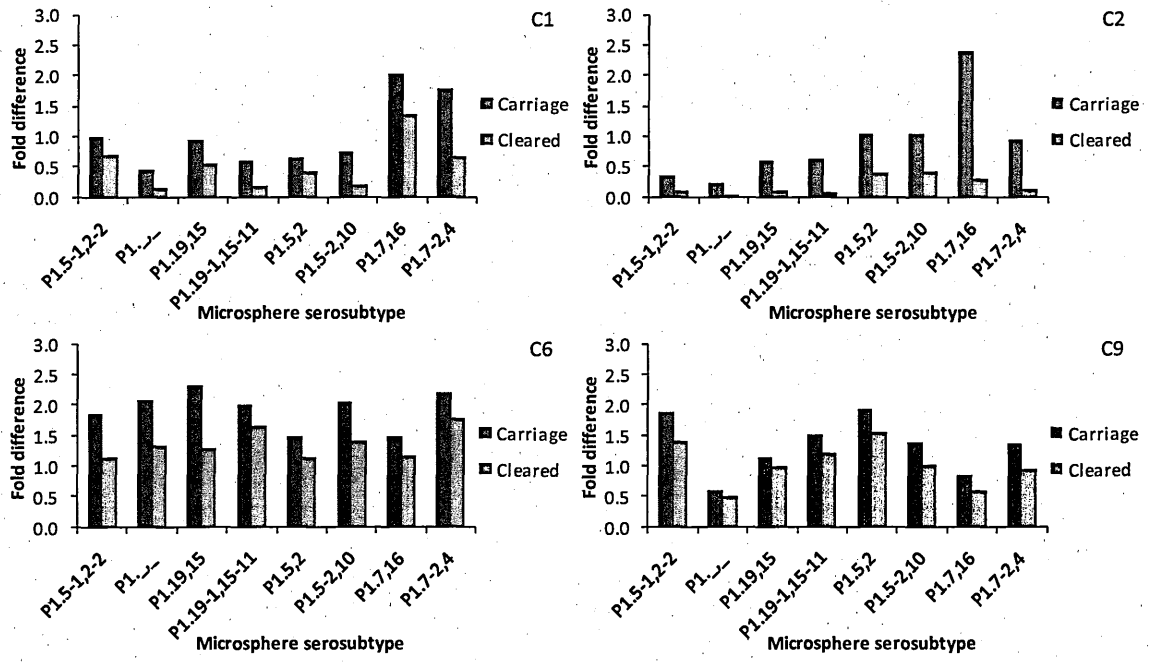


Figure 6.7 Fold difference between time points for individuals known to have cleared carriage of meningococcal isolates. Sera responses to specific serosubtypes was determined following analysis with the Liquichip assay. IgG titres were determined using a standard curve composed of pooled sera from healthy laboratory workers vaccinated with either the P1.7,16 or the P1.7-2,4 OMV vaccine. Three samples were analysed for each individual in order to determine increases in serosubtype specific IgG responses both prior to and post clearance of the meningococcal isolate. Samples were collected over a 24 week period for each individual with the exception of C9 where t = 12 weeks. Individuals C1 and C2 were found to have cleared carriage of a meningococcal isolate with the P1.21,16 serosubtype. C6 and C9 were found to have cleared carriage of meningococcal isolates with the P1.5-1,10-1 and P1.5,2 serosubtypes, respectively. Where the homologous serosubtype was not included in the Liquichip assay, sera responses were determined against the closest microsphere set.

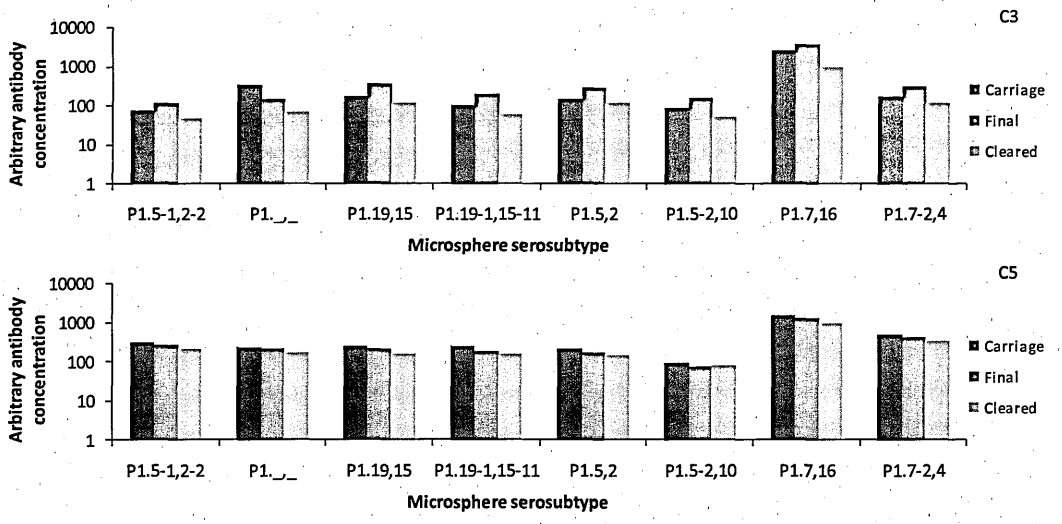


Figure 6.8 IgG titres for sera from individuals C3 and C5, known to have cleared carriage of a meningococcal isolate with a P1.21,16 serosubtype. Individuals were found to be carriers of meningococci at initial sampling, and again at t = 12 weeks, however were found to have cleared meningococcal carriage at t = 24 weeks. Whilst the homologous serosubtype was not tested, IgG responses were expected to be directed to P1.7,16 labelled microsphere. Titres were determined using a standard curve composed of pooled sera from healthy laboratory workers vaccinated with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

Elevated IgG concentrations, determined using the P1.7,16 labelled microspheres, preceded clearance of a meningococcal isolate with a P1.21,16 serosubtype for individuals C3 and C5 (Figure 6.8). Low IgG levels were found in response to all remaining PorA labelled microspheres. An increased IgG response, specific to the P1.7,16 serosubtype, was observed for individual C3 preceding clearance although this was not found to be a two fold increase. A decrease in IgG response was observed for C5.

Non-specific decreases in IgG concentrations were determined using the panel of PorA labelled microspheres for three individuals (Figure 6.9). No difference in IgG response was found prior to and post clearance of the carriage isolate for individuals C4 and C8, known to have cleared carriage of a meningococcal isolate with a P1.21,16 and a P1.5-1,10-26 serosubtype, respectively. Whilst C11 was found to have elevated IgG titres following detection with the P1.7-2,4 labelled microsphere, decreased IgG responses were observed for all other serosubtypes of PorA using the

panel of labelled microsphere both prior to and post clearance of the carriage isolate with a P1.5-1,2-2 serosubtype.

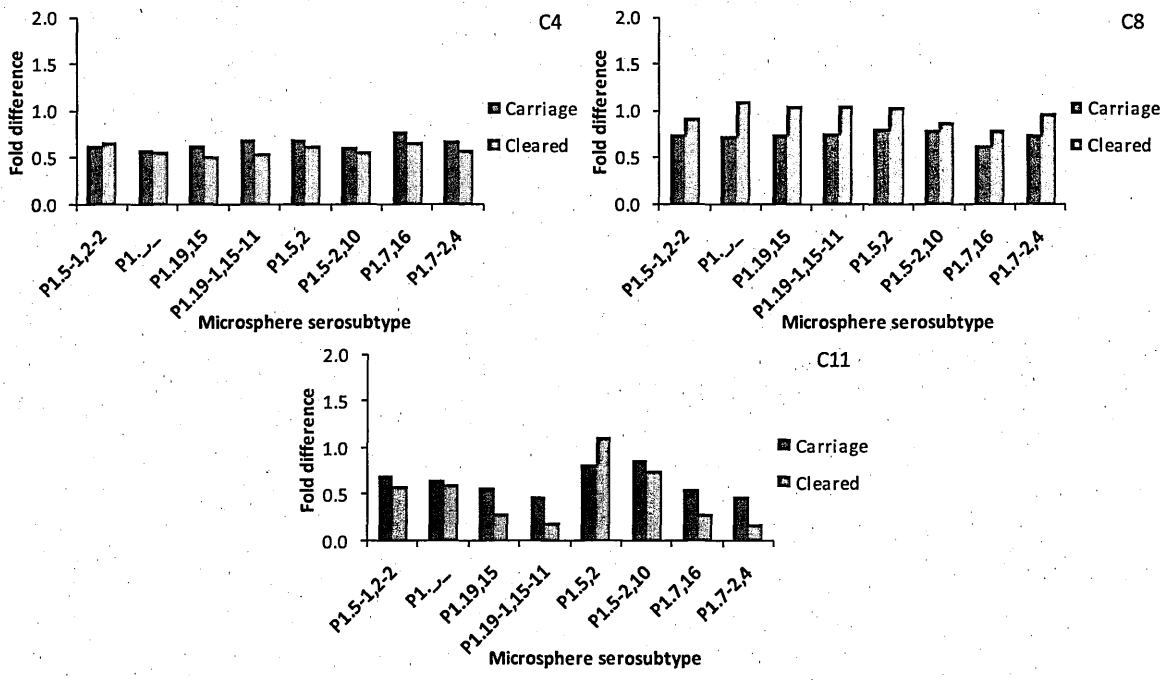


Figure 6.9 Fold difference between time points for individuals known to have cleared carriage of meningococcal isolates. Sera responses to specific serosubtypes was determined following analysis with the Liquichip assay. IgG titres were determined using a standard curve composed of pooled sera from healthy laboratory workers vaccinated with either the P1.7,16 or the P1.7-2,4 OMV vaccine. Three samples were analysed for each individual in order to determine increases in serosubtype specific IgG responses both prior to and post clearance of the meningococcal isolate. Samples were collected over a 24 week period for each individual with the exception of C11 where t = 20 weeks. Individual C4 was found to have cleared carriage of a meningococcal isolate with the P1.21,16 serosubtype. C8 and C11 were found to have cleared carriage of meningococcal isolates with the P1.5-1,10-26 and P1.5-1,2-2 serosubtypes, respectively. Where the homologous serosubtype was not included in the Liquichip assay, sera responses were determined against the closest microsphere set.

Table 6.7 Fold increase in IgG concentrations observed between samples from the same individual immediately preceding clearance of a meningococcal isolate at timed intervals, with the exception of C10 for which the post-clearance sample was used. Differences were calculated in relation to the IgG responses during periods of persistent carriage for each individual. Microsphere serosubtypes corresponding to the carriage isolate is shown in bold.

Samples	Protein labelled microspheres							
	P1.5-1,2-2	**P1._,_**	**P1.19,15**	**P1.19-1,15-11**	**P1.5,2**	**P1.5-2,10**	**P1.7,16**	**P1.7-2,4**
C1	1.00	0.46	0.93	0.59	0.64	0.75	**2.01**	1.78
C2	0.36	0.23	0.61	0.62	1.04	1.03	**2.39**	0.94
C3	1.69	0.45	2.00	2.00	1.95	1.89	**1.42**	1.92
C4	0.63	0.59	0.63	0.69	0.70	0.61	**0.78**	0.68
C5	0.85	0.90	0.86	0.77	0.82	0.82	**0.89**	0.84
C6	1.84	2.06	2.31	2.01	1.49	**2.05**	1.49	2.18
C7	3.31	2.20	1.44	3.21	2.03	**44.09**	1.31	1.31
C8	0.74	0.73	0.75	0.76	0.81	**0.79**	0.62	0.74
C9	1.87	0.60	1.13	1.50	**1.93**	1.38	0.85	1.35
C10	77.47	2.64	1.08	1.83	**94.81**	1.38	0.23	0.13
C11	**0.70**	0.64	0.57	0.47	0.80	0.86	0.54	0.46

The general trends observed using this data, appear to suggest the presence of pre-existing anti-PorA antibodies in sera, found at a low concentration in serum from individuals not found to be carriers of meningococcal isolates. Acquisition of carriage was accompanied by a greater than two-fold increase in antibody titre directed against the serosubtype of the meningococcal isolate. Specificity of this increase varied between individuals, with increases in IgG titre determined against heterologous PorA serosubtype observed with some individuals. This may suggest cross-reactivity of the antibody through the VR3 region or PorA, or the boosting of a pre-existing immune response. Increases in IgG titre were detected within eight weeks of the onset of carriage, with increasing titres observed as carriage was established. IgG titres of serosubtype specific anti-porA antibodies largely unchanged over a period of persistent meningococcal carriage, suggesting a maintenance level of antibody was achieved, sufficient to provide protection against infection, but insufficient to support clearance of the meningococcal isolate. As with acquisition of carriage, clearance of meningococcal isolates was found to be associated with a greater than two fold increase in serosubtype specific IgG titres, directed against the serosubtype of the meningococcal isolate immediately prior to clearance. However, it is important to remember the Liquichip is a measure of antibody binding, not a measure of functional bactericidal antibodies, and increases in IgG titres directed against heterologous PorA serosubtypes may not be associated with an increase in bactericidal antibodies. All sera samples were run in duplicate at two dilutions in order to achieve an antibody concentration within the linear range of the Liquichip assay.

6.3.2 Association of circulating PorA levels with increases in IgG concentration in healthy human sera from England and Wales over a 16 year period

To study the distribution of PorA serosubtype specific antibodies, serum from 500 randomised individuals, aged 15 to 19 years of age, were chosen to represent distribution of PorA specific antibodies across England and Wales. No information was available on the carrier status of these individuals and the use of randomised sera did not allow for paired samples to be analysed. These sera were used to study circulating PorA levels across the population, with 100 serum samples picked from every fourth year, from 1990 – 2006. Sera were analysed using the Liquichip assay and IgG responses were determined using the PorA-labelled microspheres. As stated previously, due to lack of an appropriate standard, it is not possible to make direct comparisons between IgG titres determined against the different PorA serosubtypes used in this assay. Trends can only be identified within the same microsphere sets.

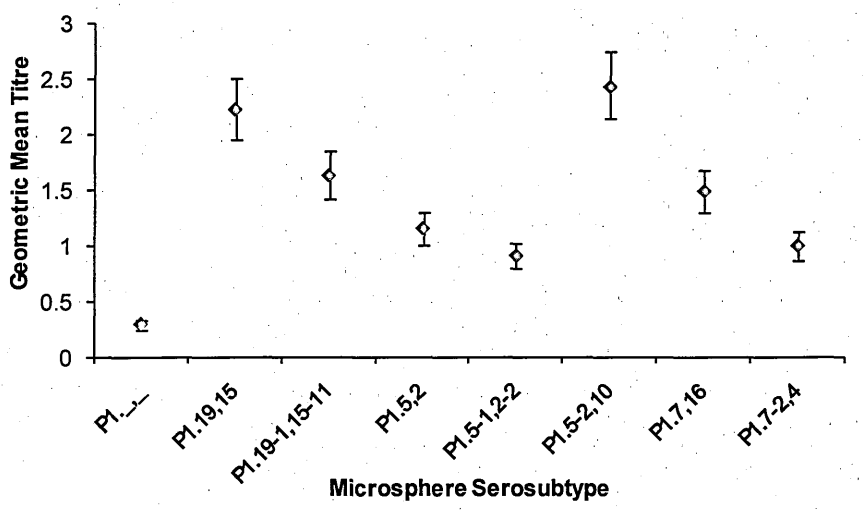


Figure 6.10 GMT in response to the panel of PorA-labelled microspheres for 500 samples from healthy individuals (error bars represent 95 % confidence intervals). Sera responses to specific serosubtypes were determined, following analysis with the Liquichip assay, using a standard curve composed of pooled serum from healthy laboratory workers immunised with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

Over a 16 year period, the lowest GMT was determined using P1._,_ labelled microspheres, whilst the highest GMT was found in response to the P1.5-2,10 serosubtype of PorA (Figure 6.10).

Strong correlation was observed between related protein variants, (P1.19,15 and P1.19-1,15-11, r=0.71; P1.5,2 and P1.5-1,2-2, r =0.69) , whilst unrelated variants correlated poorly (r ~0.1).

6.3.2.1 Geometric mean titres stratified by year

To follow the distribution of circulating PorA-specific IgG from 1990 – 2006, samples were stratified by year, with 100 sera samples used from each fourth year. An equal number of serum samples, collected from individuals from each age group, were chosen and stratified by the year in which sera was collected.

Table 6.8 GMT separated by year for each PorA variant. 100 samples were used for each time point, with sera from 20 individuals from each year of age from 15 to 19 year olds, chosen from the SEU collections. Sera responses to specific serosubtypes were determined, following analysis with the Liquichip assay, using a standard curve composed of pooled sera from healthy laboratory workers immunised with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

	Year of collection				
Serosubtype	**1990**	**1994**	**1998**	**2002**	**2006**
P1._,_	0.21	0.71	0.28	0.14	0.33
P1.5-1,2-2	0.68	**2.29**	0.51	0.54	**1.39**
P1.5,2	0.91	**2.79**	0.69	0.80	1.42
P1.5-2,10	2.60	**4.27**	1.29	1.89	**3.10**
P1.19,15	2.76	**3.66**	1.08	1.70	**2.91**
P1.19-1,15-11	1.16	**3.42**	0.88	1.41	**2.30**
P1.7,16	1.15	**3.53**	0.96	0.87	**2.12**
P1.7-2,4	0.97	**2.39**	0.58	0.68	1.06

Distribution of anti-PorA specific IgG was found to vary between 1990 and 2006 for all serosubtype of PorA used in this study (Table 6.8). Whilst the magnitude of the GMT varied for individual serosubtypes of PorA and direct comparison between serogroups cannot be made, increased titres of all antibodies specific to the panel of PorA labelled microspheres used in this study were found in 1994 and to a more limited extent in 2006. The lowest GMT observed for all serosubtype with the exception of P1._,_ in the 1998. All variants appear to show a similar

pattern in GMT over this time period. The lowest GMT, for sera collected in every year group, was observed in response to the PorA mutant P1._,_ labelled microspheres (Figure 6.11). No differences were apparent between GMTs for samples collected in 1998 and 2002 for the majority of the PorA labelled microsphere panel.

Low GMTs were determined against P1.5-1,2-2 labelled microspheres for all years from which sera samples were chosen, with a significant increase in IgG titre observed for sera collected in 1994. A secondary peak was observed for samples collected in 2006, although this was to a lesser extent than 1994, with GMTs of 1.39 and 2.29, respectively (Table 6.8). Similarly, an increase in GMT was determined using P1.5,2 labelled microspheres between 1990 and 1994, from 0.91 to 2.79, respectively. A decrease in titre, to a minimum 0.69 was observed in 1998 prior to a second increase to a titre of 1.42 for sera collected in 2006. No difference was found between GMTs for sera collected in 1990, 1994 and 2002 against either P1.5,2 or P1.5-1,2-2 labelled microspheres.

IgG titres raised in response to both these antigens would be expected to give a similar response, due to the related VR1 and VR2 regions. Disease incidence, largely attributed to the C: 2a: P1.5,2 phenotype, continued to increase to a peak in 1998/1999 and may explain the increase in GMT titres observed in 1994. Introduction of the MCC vaccine in 1999 was found to result in a large reduction of carriage of serogroup C strains of meningococci, and would account for the decrease in GMT observed in 2002. Unexpectedly, the decline in GMT against these serosubtypes of PorA were also apparent in 1998, prior to introduction of the vaccine.

GMTs, determined using the P1.5-2,10 labelled microspheres, were found to increase from 2.60, for sera collected in 1990, to a maximum of 4.27 for sera collected in 1994. A decrease in titre was again found in 1998 to a minimum GMT of 1.29, followed by an upward trend in IgG titre, with increasing GMTs in 2002 and 2006.

Elevated IgG responses to P1.19,15 labelled microspheres were found in sera collected in 1990 and 1994, with a GMT of 2.76 and 3.66 found, respectively. A decreased GMT was observed in 1998 and was followed by a small increase in titre found in 2002. A further increase was observed

in 2006, with a GMT of 2.91. Similar titres were observed for sera collected in 1990, 1994 and 2006. A large increase in GMT was determined using P1.19-1,15-11 serosubtype labelled microspheres between sera collected from individuals in 1990, with a GMT of 1.16, and 1994 with a titre of 3.42. A decrease in GMT to a minimum of a 0.88 was observed in sera collected in 1998, followed by a second increase in IgG concentration in 2006.

Responses determined against both P1.7,16 and P1.7-2,4 labelled microspheres followed a similar trend. The maximum GMT, observed for both serosubtypes, was found in sera collected in 1994, with titres of 3.53 and 2.39, respectively. High frequencies of these serosubtypes were recorded in the UK in 1995[340]. Decreased titres were observed in 1998 and 2002, prior to a second increase in IgG titre in 2006, though this increase was to a more limited extent in response to P1.7-2,4 labelled microspheres.

Disease incidence attributed to meningococcal isolates expressing the P1.7,16, P1.5-2,10, P1.19,15 and P1.19-1,15-11 serosubtypes of PorA have been in decline since 1993 as a result of herd immunity[126] and may explain the increased GMT against these serosubtypes observed in 1994. Whilst reduction of carriage with the MCC vaccine resulted in reduction in carriage of serogroup C meningococci, reduction in serogroup B meningococci was not observed. Serogroup B meningococci have shown a higher phenotypic diversity than serogroup C isolates, and may explain the increase GMT determined observed against all serosubtypes of PorA in 2006. The presence of a peak GMT observed for all serosubtype of PorA in 1994 was not expected and known outbreaks have not been reported for that period. As previously stated, GMT are a reflection of total binding activity in sera, and not restricted to functional bactericidal antibodies.

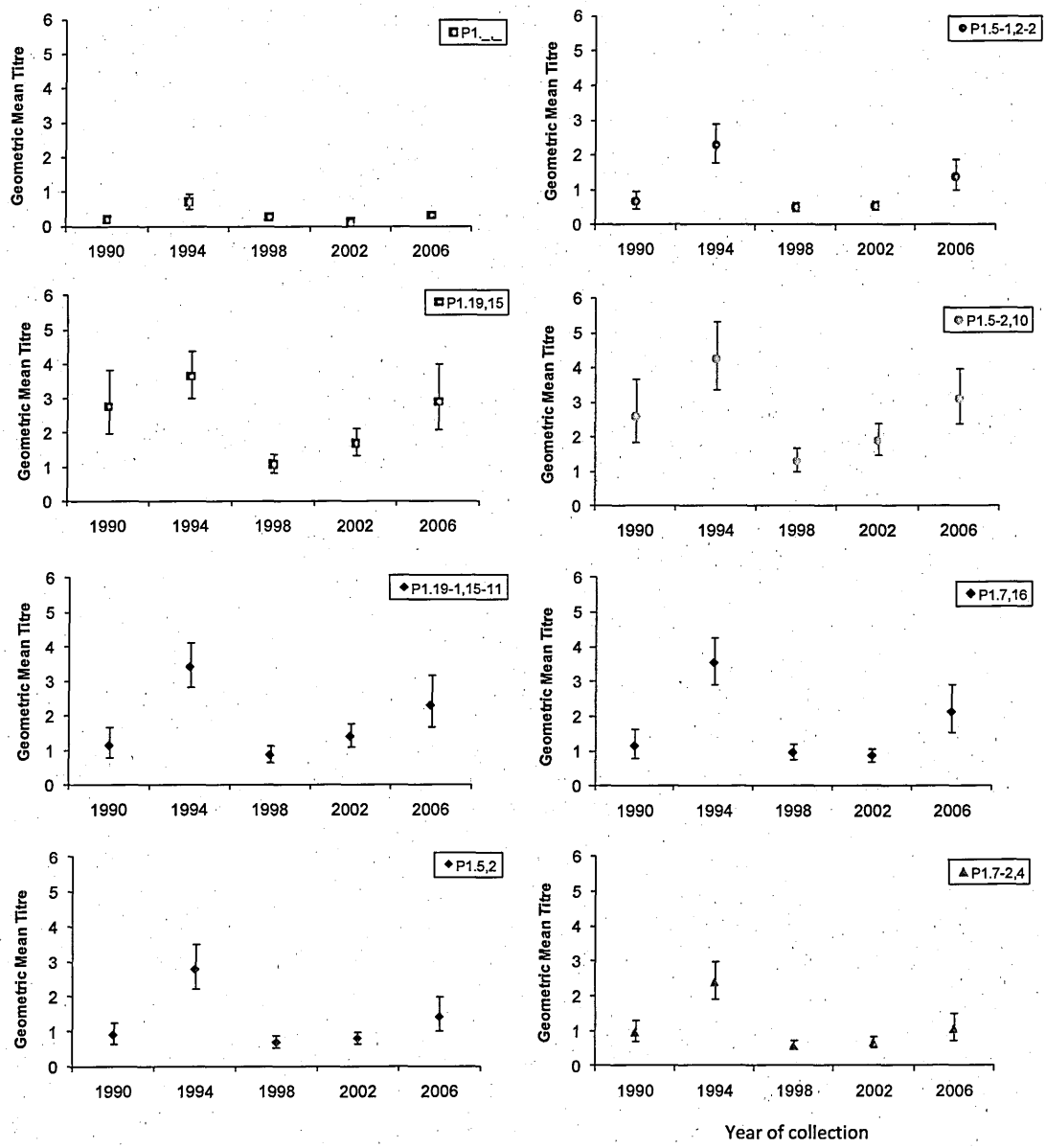


Figure 6.11 GMTs of IgG responses separated by year for each PorA variant (error bars represent 95 % confidence intervals). 100 samples were used for each time point, with 20 individuals from each year of age from 15 to 19 year olds, chosen from the SEU collections. Sera responses to specific serosubtypes were determined, following analysis with the Liquichip assay, using a standard curve composed of pooled sera from healthy laboratory workers vaccinated with either the P1.7,16 or the P1.7-2,4 OMV vaccine.

6.4 Discussion

The overall aim of the studies described in this chapter was to investigate the effect of meningococcal carriage on the serum IgG titres in different groups of individuals and potential changes in exposure to meningococcal isolates over time.

Carriage studies are of importance in providing information about the epidemiology and pathogenesis of meningococcal disease, and in the identification of risk factors for carriage. Closed communities such as military camps and college students, with increased levels of meningococcal carriage provide an environment in which to study the dynamics of meningococcal carriage and the immune response to carriage[152]. A longitudinal study was used to study the effect of meningococcal carriage in first year university students over a period of up to 24 weeks. The carriage state of each student was determined as part of a separate study and the serosubtype of the meningococcal carriage isolate was identified, where applicable[25]. This allowed analysis of developmental trends and direct comparison between serum IgG concentrations and known carriage states. There are several stages involved in acquisition of meningococci including non-carriage, first encountering organism, persistent carriage, and clearance of carriage, all of which occur without symptom. As described previously, an increase in serosubtype specific antibody response can be used as an indicator in the evaluation of vaccine response and can also be used to determine carriage status of individuals. Carriage of meningococcal isolates tend to lead to the induction of SBA, however this assay measures binding concentrations of both functional and non-functional antibodies. Serum IgG levels were assessed using the Liquichip assay developed during the earlier part of this project. The use of multiple samples from the same individual allowed changes in IgG titres to be measured as both arbitrary concentrations and as an increase from the initial time point.

The lowest IgG concentrations were directed towards microspheres labelled with the P1._,_ PorA mutant in which VR1 and VR2 had been removed. As observed in previous chapters, the low MFI values determined in the standard sera using the P1._,_ labelled PorA protein would have

resulted in elevated IgG titres. This suggested that anti-PorA antibody responses are largely specific to the VRs and not directed to the protein backbone of PorA, in contrast to the results observed with vaccinated sera in chapter 5 of this thesis. However, as noted in previous chapters, the analysis of antibodies in normal human sera is complex, with antibodies produced in response to whole meningococci as opposed to specific proteins and antibody responses of different individuals would be expected to differ as a result of previous carriage[364], even after clearance of the meningococcal isolate.

Non-carriers had low IgG responses to the panel of PorA labelled microspheres. This is consistent with previously published results in which individuals with a low antibody response to all serosubtypes of PorA were associated with non-carriers[254,152]. The presence of low IgG responses determined in serum from non-carriers, as opposed to a lack of response, may be indicative of previous carriage. Whilst carriage of meningococcal isolates was not detected at the time of sampling, it is not known whether previous carriage of meningococcal isolates has occurred[153].

New carriers were shown to exhibit a greater than two fold increase in serosubtype specific antibody concentration. This increase in antibody production was likely to have been stimulated by the presence of carriage isolates[152] in agreement with previously published data whereby meningococcal carriage is an immunising event resulting in the development of specific antibodies, homologous to the carriage strain[133]. For the individuals in this study, an increase in titre of serosubtype specific antibodies was observed within eight weeks of the onset of carriage, whilst increases in antibody titre as early as two weeks following the acquisition of a meningococcal carriage isolate have previously been reported[114] Direct correlation was found between the amount of time that had elapsed between the gain of carriage and the time at which sera was collected, and the extent to which antibody responses were increased. The highest antibody titres were recorded at the furthest time point, up to 24 weeks following acquisition of carriage as shown by Individuals N3 and N5 (Figure 6.2). An increase in IgG concentration in response to all panel PorA serosubtypes was observed for individual N5, in particular the P1.19,15

labelled microspheres. This may have been a result of previous carriage and clearance of meningococcal isolates with this serosubtype immediately prior to collection of the initial sera sample or the presence of antibodies binding to parts of the PorA protein other than the VR1 and VR2. This was demonstrated with the use of the PorA mutant (P1._,_). This protein was expressed as a PorA protein lacking VR1 and VR2, and pre-inhibition with this protein may prevent binding to epitopes other than the VR1 and VR2 of PorA. Some activity has been demonstrated against the VR3 region of PorA, and variants of the P1._,_ protein expressing differing VR3 epitopes would have to be cloned. Elevated levels of PorA antibodies, produced in response to carriage are likely to play a role in the development of natural immunity to meningococcal disease[152].

Specific IgG concentrations were found to be largely unaffected over the 12 - 24 week reporting period where persistent carriage was observed. Persistent carriage was not found to have a serosubtype specific effect on antibody titres with increases or decreases in IgG titres observed against many of the PorA IgG serosubtypes tested. In contrast to new carriers of meningococci, longevity of meningococcal carriage in persistent carriers was not found be associated with IgG titres and little difference was observed against anti-PorA antibody titres in sera collected at t = 12 weeks and t = 24 weeks This suggested that this concentration of antibody acted as a maintenance level sufficient to protect against infection, but insufficient to support clearance of the carriage isolate [133] and carriage may persist over many months[8].

Clearance of carriage isolates, as with acquisition of the meningococcus, was associated with a large increase in serosubtype specific IgG titre. This was found to be in the form of a greater than two fold increase in titre (Figure 6.6 and Figure 6.7), or elevated IgG titres (Figure 6.8) observed immediately prior to clearance followed by a reduced titre once the carriage isolate could no longer be detected. This suggests that these largely elevated levels of serosubtype specific antibodies resulted in an antibody concentration sufficient to clear meningococcal carriage, with responses to the remaining serotypes remaining largely unaffected. As the Liquichip multiplex

assay is an antibody binding assay, a functional antibody assay, such as the SBA assay would be needed to determine whether this increase was due to the production of bactericidal antibodies.

Priming of the immune system through the use of vaccination would have a direct effect on the rate at which meningococcal carriage is cleared and the presence of circulating antibodies has been suggested as a more appropriate correlate of protection against meningococcal disease[33] than the ability to generate a memory response[14]. However the impacts of meningococcal vaccines on carriage rates have been varied. Polysaccharide vaccines were found to have little effect on carriage rates due to the rapid decline of antibody titres raised in response to these vaccines and insufficient antibody titres to prevent colonisation[135]. Introduction of the MCC vaccine was found to induce herd protection and a 66 % reduction was observed in nasopharyngeal carriage, in students aged 15 to 18 years of age, specific to both serogroup and clonal complex within the first year[187]. However the vaccine was not found to have an effect on the other serogroups, in particular, the prevalence of carriage of meningococci most commonly associated with serogroup B disease (members of the ST-41/44 complex) was unaffected by the introduction of the MCC vaccine[186]. With a lower prevalence of disease and carriage in vaccinated compared to unvaccinated individuals, benefits to older age groups have been observed with a significant herd immunity effect in those that have not been vaccinated with conjugate vaccines[187]. The use of OMV vaccines was found to result in reduced carriage rates and correlation has been demonstrated between anti-PorA antibody responses in sera from individuals vaccinated with an OMV vaccine and bactericidal activity[346]. Although vaccination can be used to prime the immune system, the rapid development and progression of meningococcal disease, means the stimulated response from immune memory may not occur quickly enough to protect against infection[228]. Therefore the presence of circulating antibodies may be critical for clinical protection against disease[33,82].

Analysis of Seroepidemiology samples

A seroepidemiology study was carried out using sera obtained from the HPA SEU to study the prevalence of serum anti-PorA antibodies in sera collected from 15 – 19 years olds around England and Wales over a 16 year period. These samples had been collected as part of clinical investigations for medical conditions other than Meningococcal disease and carriage status was unknown.

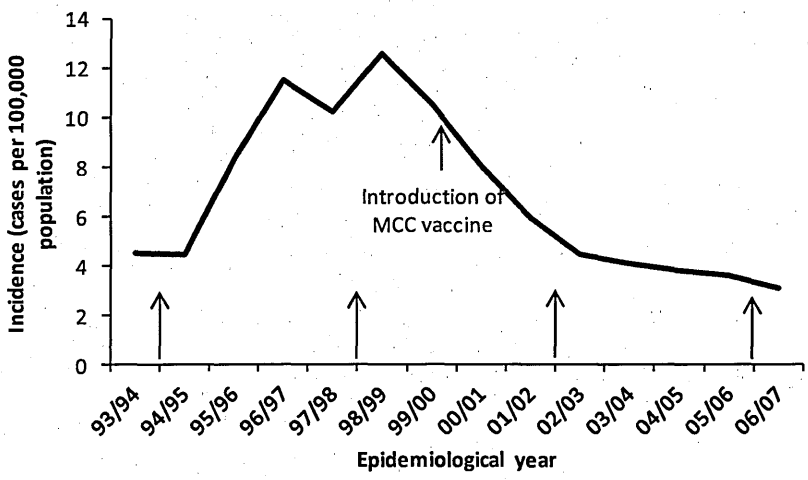


Figure 6.12 Incidence of invasive meningococcal infection in 15 – 19 year olds in England and Wales (cases per 100,000 population). Data adapted from the epidemiological data reported by the HPA. The years from which sera samples were selected are shown by the arrows.

A peak in GMT was seen against all PorA serosubtype used in this assay, in 1994, with a smaller peak observed in 2006 and show inverse correlation with the incidence of meningococcal disease reported in this age range (Figure 6.12), with high GMT detected during periods of low disease incidence. Similar patterns in the GMT were detected in all the serogroups and may have occurred as a result of using geometric mean titres whereby data was pooled and the results were homogenised. The use of such methods, whilst allowing determinations in trend, can also result in the loss of ability to resolve individual responses to specific serosubtypes. In particular, with a 25 % carriage rate estimated in 15 – 19 year olds[43,49] it is possible that high titres of anti-PorA serum antibodies present in few individuals show results determined within the overall group. Increased titre of anti-PorA antibodies detected in 1994 may have preceded an increase in disease

association in 1995 and proportionately more disease cases were found to occur in the 15 – 19 year age group following an age shift between 1994/95 and 1995/96[126]. The GMTs detected using the Liquichip assay were found to have declined for all anti-PorA antibodies in the 1998 sera samples, corresponding with an increase in disease incidence that continued to a peak in 1998/1999[126]. The increase in disease was largely attributed to the Serogroup C cases, due to the C: 2a: P1.5,2 phenotype however this is not reflected in GMT determined using the P1.5,2 and the P1.5-1,2-2 PorA labelled microspheres. Decreased antibody titres specific for the P1.5,2 and P1.5-1,2-2 proteins observed in 1998 and 2002 reflect the decline in disease association resulting from introduction of the MCC vaccine[359,246,126,282]. Whilst introduction of the serogroup C conjugate vaccine was found to result in a large reduction in carriage of meningococcal serogroup C strains, this effect was not observed for other serogroups[187], and may go some way to explain the secondary peak in 2006 with increased carriage of non-serogroup C meningococcal strains. A slight increase in meningococcal disease attributed to serogroup Y has been reported since 2007, and again may have been preceded by an increase in IgG titres to specific PorA serosubtypes detected using this assay.

The most common cause of meningococcal disease are Serogroup B meningococci, representing up to 69 % of disease cases in 1993/94, 46 % in 1996/97 and increasing to 87 % following introduction of the MCC vaccine. Serogroup B isolates have shown higher phenotypic diversity of serogroups than serogroup C isolates and a number of changes in strain population have been recorded over the assay period[126]. GMT titres in response to the P1.7,16, P1.5-2,10 P1.19,15 and P1.19-1,15-11 PorA labelled microspheres were determined to have a similar GMT profile as observed with the P1.5,2 serosubtype PorA protein. Disease incidence attributed to meningococcal isolates expressing each of these serosubtypes have been shown to be in decline since 1993 possibly due to an increase in host/herd immunity[126]. This natural immunity may account for the increase in GMT detected for sera samples collected in 1994 and in 2006, as previously stated, the Liquichip assay measures binding antibodies and not just bactericidal antibodies. This was further supported by the gradual accumulation of immunity to the

meningococci expressing the P1.7-2,4 serosubtype of PorA following the predominance of strains expressing the P1.4 serosubtype.

Chapter 7. General Discussion

7.1. Discussion

The most straightforward method of assessing immune responses against vaccines is the measurement of serum IgG titres directed against the vaccine. These methods alone are not a good measurement for the effect of vaccination alone and whilst IgG titres are a measure of antigen binding antibodies, the production of bactericidal antibodies cannot be conferred. Methods such as the SBA would also need to be performed for vaccine assessment, and the ability of the vaccine to produce a protective response. Whilst current ELISA based methods are well suited for the detection and quantification of serum IgG antibody titres against single analytes, the same cannot be said for multiple analytes. This requirement for multiple ELISA is both time consuming and costly in terms of serum volume for multiple analytes[167]. A microsphere based multiplex immunoassay assay, such as the one described here, has several advantages over standard ELISA. In theory, antibodies raised against 100 different antigens can be measured simultaneously due to the unique fluorescent signature of each microsphere set. The ability to multiplex the measurement of antibodies to multiple antigens within a single reaction resulted in a decrease in processing time, and a reduction in the volume of reagents and sera required for analysis in comparison to the standard ELISA[169]. This is of particular importance in instances such as with the use of infant sera which may only be available in minute quantities. With the introduction of multivalent vaccines, such as Hexamen and Nonamen, the detection of multiple antigens within a single assay is advantageous. These vaccines have been found to be safe and immunogenic with responses to all of the vaccine serosubtypes detected. A new generation of vaccine has been produced based on Nonamen, utilising the detergent free extraction of OMVs, used to preserve the native (attenuated) LPS content and retains protective lipoproteins[154]. As with Nonamen, this vaccine is based on three trivalent OMVs and has been shown to induce high SBA titres against all PorA serosubtypes included in the vaccine strain, and also elicited cross protection against meningococcal B strains with non-vaccine PorA serosubtype in pre-clinical trials[315,154].

Although no comparison was made between the multiplex PorA assay and ELISA in this thesis, previous studies have shown strong correlation between multiplex immunoassays and ELISA using both meningococcal and pneumococcal capsular polysaccharides[229,230,78,167,168,242,94,67,194]. Studies have also been performed to evaluate the use of a microsphere based multiplex assay for antibody response to organisms including: Hib bacteria; measles virus, mumps virus and rubella virus; Diphtheria toxin; pertussis bacterium; Clostridium tetani (the Tetanus causing bacteria); and Hepatitis C virus[66,96,285,326,278]. The small volumes of serum required along with the time and cost effectiveness of a multiplex assay in relation to the more traditionally used detection methods are highlighted in each case.

In this thesis a multiplex immunoassay, based on Luminex technology was developed and validated for the simultaneous detection and quantification of serum IgG responses to a panel of seven serosubtypes of PorA along with a VR1/VR2 deleted mutant. Assay suitability was determined using pre-clinical trial sera; clinical trial sera; and sera from carriage studies. The developed assay was found to be specific, sensitive and reproducible. Several variants of target antigens were amplified, cloned and used to express of protein resulting in the purification of eight PorA proteins, three PorB proteins and five FetA proteins as described in Chapter 3. Four variants of the *fHbp* were also cloned but were not used for protein expression as a result of time constraints. All of these proteins can be used as antigens within the liquichip assay, but only the PorA proteins have been used in this study.

For the purpose for this study, highly purified PorA proteins, established to include the PorA serosubtype expressed by the most commonly circulating meningococcal isolates were used to create a panel of PorA-labelled microspheres for use in the development of the microsphere based immunoassay described in this thesis. A VR1/VR2 (P1._,_) deleted mutant was also included in this panel to detect the presence of antibodies binding to regions of PorA other than VR1/VR2. His-tagged PorA proteins were conjugated to nickel coated microspheres ensuring that the proteins immunogenic epitopes were accessible to serum antibodies, this was confirmed using monoclonal antibodies, specific to the VR1 and VR2 epitopes of PorA. An important

consideration for multiplex assays is the possibility of interference or cross-reactivity [167]. However, the use of mouse monoclonal antibodies demonstrated no evidence of cross-reactivity or interference was found to exist between the microsphere sets and the assay was found to be equally sensitive whether used for the detection of a single or multiple analytes. This was consistent with other studies on the multiplex immunoassay[167,67]. Minimal intra- and interassay variation was found when repeat samples were analysed and the resulting CVs were comparable to those previously reported for multiplex immunoassays[40,167]. Assay specificity is of particular importance in the development of any multiplex assay as this may result in the reporting of false negative, or false positive results. Specificity of the multiplex PorA immunoassay assay was demonstrated with the use of MAbs and cross-reactivity was only observed between related PorA variants. This was further supported by inhibition experiments using MAbs incubated with purified proteins. The assay was not found to be as specific using human adult serum and a reduction in the reactivity of human serum was observed following inhibition studies with recombinant proteins, on both homologous and unrelated serosubtypes. However, this cross-reactivity can be explained by the presence of pre-existing antibodies in human sera. Since humans are carriers of meningococci, the presence of anti-meningococcal antibodies in adult sera will influence the immune response initiated in response to a new infection or vaccination[113]. Non-specific binding of human serum antibodies directly to the beads has previously been suggested[339], however, the use of the VR1/VR2 deleted mutant labelled microspheres showed this was not the case in this study.

As described in this thesis, the multiplex PorA assay was successfully used for the detection of increases in serum IgG titres against specific PorA serosubtypes following vaccination with one of four different OMV vaccines in preclinical and clinical trial sera. Increased GMTs were determined against all PorA serosubtypes included in the multiplex assay panel using murine and human toddler/child sera following vaccination with either HexaMen or Nonamen[316]. Low antibody titres were detected in pre-vaccination and unvaccinated control sera confirming the absence of pre-existing anti-PorA antibodies. Antibody responses detected in the post-vaccination sera are

therefore likely to have been produced in response to vaccination. The highest GMT was detected in sera following the booster dose of the vaccine, with similar responses determined following vaccination with three doses of either the HexaMen or the NonaMen vaccine. Although only a small number of samples were analysed, the vaccine response was variable between individuals, with high antibody titres determined against all PorA serosubtypes in one serum sample following one dose of the HexaMen, and titres lower than pre-vaccination sera titres in another. This highlighted the need for analysis of pre- and post-vaccination sera from the same individual, to allow for the vaccine response to be determined particularly where sample size is small.

Increased IgG titres were also determined against specific PorA serosubtypes in human adult sera immunised with both the P1.7,16 or the P1.7-2,4 OMV vaccines as previously reported[267]. However, as previously mentioned, immune responses were found to be dependent on the individual rather than the vaccine with the presence of high and low responders to the OMV vaccines. The use of monoclonal antibodies found the assay to be specific with no cross reactivity observed between microsphere sets. Inhibition with the P1._,_ protein was not found to affect the antibody responses determined against the heterologous PorA labelled microspheres, with the exception of the P1.7,16 and P1.7-2,4 serosubtypes of PorA, where a slight decrease was observed, and is likely to be due to the VR3 epitope of PorA homologous to these proteins. This confirmed the majority of antibody binding was directed against VR1 and VR2 epitopes. As with the inhibition study, the presence of pre-existing antibodies in adult sera, detected using the PorA assay, may have appeared as non-specific responses to the vaccine, or may have been boosted following vaccination. It is likely that increasing the scale of this study would have resulted in findings similar to those previously reported[92,267].

This assay is likely to be useful in the evaluation of complex vaccine formulations containing a number of different PorA serosubtypes. The Liquichip assay can therefore be tailored for the assessment of individual vaccines, with only the serosubtypes of interest included in the protein labelled microsphere panel. The multiplex assay can easily be adapted for use in any number of

studies with flexibility in the number of PorA serosubtypes to be tested and the serosubtype variants used within the assay. Use of an assay such as this has a valuable role in determining the effects of vaccination, not only on the PorA serosubtype homologous to the vaccine strain(s) but also on heterologous PorA antigens within a single reaction thereby allowing determination of the cross-protective effect of vaccination on any number of PorA serosubtypes.

An alternative use for this assay in addition to vaccine evaluation, would be of use for immunological studies of meningococcal carriage with antigenic variants of interest included in the microsphere panel. Due to the high genetic diversity of PorA, and the changing serosubtype prevalence of meningococci, it is necessary to monitor circulating strains, particularly in regions where the vaccine strain may need to be adapted[9]. However the current method for determining meningococcal carriage is time, reagent and labour intensive and may explain why current carriage studies in Europe are limited, particularly at a serosubtype level. The ability to adapt the PorA multiplex immunoassay to include up to a 100 variants of interest can be of benefit both for longitudinal and cross-sectional carriage studies resulting in the high throughput of sera in a time and cost effective manner with a reduction in the volume of sera required. This was shown through the use of the PorA multiplex assay to perform immunological analysis on carriage samples collected as part of a study to evaluate the effect of meningococcal carriage in first year university students over a period of up to 24 weeks[25]. Differences in sera immune response determined against the panel of PorA serosubtypes highlighted the known carrier state of individuals, with direct comparisons able to be made between IgG titres and known carrier states. Low IgG titres were found in sera from individuals determined to be non-carriers of meningococcal isolates consistent with previously published results[254,152]. New carriers were shown to exhibit a greater than two fold increase in serosubtype specific antibody concentration, possibly stimulated by the presence of carriage isolates[152]. Increased IgG titres were found to correlate with the amount of time that had elapsed between gain of carriage and the time at with sera was collected. Elevated levels of PorA antibodies, produced in response to carriage are likely to play a role in the development of natural immunity to meningococcal disease[152]. Persistent

carriers of the same meningococcal isolate were not found to have a correlation between longevity of carriage and IgG, with little difference observed in IgG titre over time. It has been suggested that this concentration of antibodies acted as a maintenance level[133]. The highest increase in serosubtype specific IgG titres was determined in individuals preceding cleared carriage of meningococci with a reduced IgG titre following clearance, with IgG titres to the other PorA serosubtypes largely unaffected. The Liquichip is a binding assay and as such was not able to determine the presence of functional antibodies following a period of carriage.

Suitability of the PorA multiplex assay for seroepidemiology studies was demonstrated using sera samples collected over a 16 year period across England and Wales, however mixed results were obtained from this study. Increases in specific anti-PorA antibody titres were found to correlate well with increased disease incidence[126]. In particular a peak in disease incidence in 1998 was largely attributed to the Serogroup C meningococci, with the C: 2a: P1.5,2 phenotype and was reflected in the increased GMT determined using the P1.5,2 and the P1.5-1,2-2 PorA labelled microspheres. Decreased antibody titres specific for the P1.5,2 and P1.5-1,2-2 proteins observed in 1998 and 2002 reflect the decline in disease incidence reported in England and Wales over this period (Figure 6.12) this is particularly evident following introduction of the MCC vaccine in 1999[126,282]. Similar trends were observed in the IgG titres against all serosubtypes of PorA, however with a decline in disease incidence attributed to meningococcal isolates expressing each of these serosubtypes[126], natural immunity may account for the increase in GMT detected for sera samples collected in 1994 and in 2006 corresponding to a decrease in disease incidence, however as previously stated, the Liquichip assay measures binding antibodies and these may not be bactericidal antibodies.

There are many advantages of Liquichip assay in comparison to use of the ELISA, not least of which is the reduction in the amount of time, reagents, and sera required for the analysis of IgG responses to multiple antigens within a single serum sample. Flexibility of the Luminex system has allowed for the conjugation of different types of biological molecules to the microsphere including capsular polysaccharides, lipopolysaccharides, cytokines and nucleic acids and assays

developed using this molecules have been the subject of many studies[229,230,78,167,168,242,94,67,194,53]. Whilst the majority of meningococcal studies performed using the multiplex assay have been based on the capsular polysaccharides[167,168,67], the assay described in this thesis focused on the detection of serum antibodies specific to the outer membrane protein, PorA. This is of particular importance following the development of vaccines based on components of the meningococcal outer membrane as opposed to capsular polysaccharides. Use of the multiplex assay ensures the immune response to multivalent vaccines can be determined without the need for large volumes of serum and therefore well suited for the evaluation of vaccine immunogenicity in infants and small children. The main advantage over a multiplex assay such as this over the more traditionally used ELISA is adaptability of this system to evaluation response to any number of antigens without increasing specimen volume or processing time[168] and a multiplex immunoassay has been developed to evaluate serum response to 14 capsular polysaccharides[30].

The liquichip assay was found to be a useful tool in the evaluation of vaccine, however it cannot be used as a functional assay, and the effect of vaccination on bactericidal antibodies would have to be measured using a SBA assay. SBA values have long been used as a measure of vaccine efficacy however good correlation between antibody titres determined using ELISA and functional activity determined using SBA has not always been reported. Good correlation between SBA and ELISA in sera from adults, toddlers and infants was reported following immunisation with the MCC vaccine[277]. However poor correlation was reported in infant sera following vaccination with a polysaccharide vaccine, with no bactericidal activity detected despite high serum antibody responses[176,197]. Whilst antibody dependant bactericidal activity will be reflected in the responses determined using the Liquichip assay, antibody independent activity will not. IgM antibodies may also contribute to SBA activity, and again cannot be not quantified using the assay develop in this study. SBA values for the sera used in this study would have been useful in the evaluation of non-specific responses in sera for both vaccine, and carriage studies, and provided a useful link between vaccine response and non-protective antibodies. Whilst the vaccines used in this study are largely based on the immunogenicity of PorA, the effect of vaccination on IgG titres

to other meningococcal antigens would be advantageous, particularly with the OMV vaccines. Addition of the PorB and FetA antigens to the panel of protein labelled microspheres would allow further evaluation of the vaccines on individual IgG responses, and alongside SBA would be useful for determining the effectiveness of a vaccine to produce a broadly protective response. In England and Wales, peaks in meningococcal disease have been associated with meningococcal isolates expressing either the PorB 2b or the 2a serotype in 1973-75[240], isolates expressing the PorB 15 serosubtype in the 1980s[240], and more recently meningococci isolates expressing the serotype 4 of PorB[48]. Use of the Liquichip to determine IgG titres against these serotypes could be a useful tool for the determination of trends in the prevalence of meningococci isolates expressing different serotypes of PorB

The high sensitivity and specificity of the assay has allowed for the discrimination of antibody responses to several antigenic variants of the PorA protein. The use of pooled mouse monoclonal antibodies can be used for the quantification of antibodies in murine serum. However, comparison of immune responses to the different serosubtypes is hindered by the lack of a suitable human reference serum and known antibody levels within the reference serum. For the purpose of this thesis, sera collected from healthy laboratory workers immunised with three doses of the P1.7,16 or the P1.7-2,4 OMV vaccine were pooled for use as a human standard. Sera from these individuals were assumed to contain high levels of IgG antibodies against a wide range of PorA serosubtypes, as a result of both vaccination and routine exposure to meningococcal isolates within the laboratory. This was confirmed using the PorA assay with the detection of specific anti-PorA antibodies against all PorA serosubtypes included in the microsphere panel. However, due to the lack of an appropriate standard the concentration of serosubtype specific anti-PorA antibodies could not be quantified. An arbitrary antibody concentration was assigned for the level of IgG antibodies determined against each PorA serosubtype in the pooled sera. All human sera antibody responses determined using this assay were quantified in relation to this standard. As a result, direct comparison between the IgG concentrations determined against each of the PorA serosubtype could not be made. Where available, the use of sera collected both

prior to and post vaccination or episode of carriage helped to overcome this issue with differences in pre- and post-sera IgG responses used to compare the effect on serosubtype specific anti-PorA IgG concentrations. However the effect of vaccination or carriage on an individual could not be made where pre- and post- serum was not available.

The lack of commercially available antigen coated microspheres and the high cost of reagents required for assay development are limitations to this assay. Production and subsequent validation of both specificity and sensitivity must be performed both in the capacity of a monoplex and multiplex assay prior to use of the assay[285]. However, the advantages of an assay such as this outweigh the limitations with significant savings both in time, reagents and sera once the assay has been established.

Protein refolding could not be analysed, due to use of Triton X-100 in the purification process, however the correct conformation of the VR1 and VR2 epitopes were assumed with the use of monoclonal antibodies specific to these regions of the PorA protein. In order to analyse this more fully, proteins would need to be purified using an alternative solubilisation reagent, such as Octyl glucoside, octyl thioglucoside or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) all of which can easily be removed from final purified protein extracts using dialysis[11,12].

Further development

The first step in using the assay to its full potential would be the development of an extended panel of antigen labelled microsphere sets which can be selected for use in the assay. With the high antigenic diversity of PorA, and the availability of 100 different microsphere types, all of which can be included within a single assay, expansion of the protein labelled microsphere panel to include further serosubtypes of PorA would be of benefit. The production of such a diverse panel of PorA labelled microspheres would allow for the development of tailor-made assays to be used for seroepidemiology studies of meningococcal carriage and the evaluation of vaccines containing multiple serosubtypes of PorA. Determination of vaccine induced sera IgG responses could be limited to vaccines strains, or expanded to include other serosubtypes of interest in

order to determine the cross-protectiveness of the vaccine without the need for extra sample volume or time. The use of synthetic peptides corresponding to the variants of PorA VR1 or VR2[199] to label microspheres would allow more detailed analysis of immune responses induced as a result of vaccination or carriage. In addition to PorA, further development of this assay can be performed to include the use of alternative antigens including PorB, FetA fHbp and other vaccine antigens either in combination with the PorA multiplex assay or as a separate assay. As previously described, the detection of immune responses against multiple antigens has not been found to affect the outcome of the assay. IgG concentrations determined against pneumococcal and meningococcal capsular polysaccharides were not affected following the addition of meningococcal polysaccharide labelled microsphere sets into a multiplex assay for detection of immune responses directed towards pneumococcal capsular polysaccharides[168].

Further development of the work presented would include expression and characterisation of the fHBP proteins cloned in Chapter 3, preferably in the absence of Triton X, in order to allow protein refolding studies, such as to be performed. These proteins along with recombinant his-tagged proteins produced using five antigenic variants of FetA, and three variants of PorB could be used to label microsphere beads for use in the Liquichip assay. These variants are representative of those that are commonly found in the UK (FetA F1-5, F3-3, F3-6, F3-9, F5-1, and PorB 2a, 4 and 15) and those that are representative of hyper invasive lineages[314]. Following validation, this assay could be used in a similar experiment to the PorA assay, to determine the contribution of different antigenic variants to the immune response and used to expand the work described in thesis to include greater sample numbers for the evaluation of HexaMen, NonaMen, MenBvac and MeNZB and Seroepidemiology studies.

Further work in the development of this study, would also include production of highly purified his-tagged proteins to be used as antigens in the Liquichip assay and would include the use of surface membrane proteins including those identified using reverse vaccinology, such as NadA, NHBA and other components of the OMV. These proteins are currently in use as vaccine antigens and development of an assay to determine immune responses following vaccination with these

antigens could be used in combination with SBA to determine vaccine efficacy. If the project was started now, these would be the antigen targets selected, due to their inclusion in the 4CMenB vaccine. Priority for the selection of antigens used in this assay must be given to the antigen includes in vaccines currently in development as this is where the assay is most likely to be of use. Production of a library of protein labelled microsphere could then be used to develop a vaccine specific assay, with the inclusion of vaccine strains or non-vaccine strains for each antigen included as required.

Expansion of the experiments performed in this study to include larger sizes for both carriage and vaccine studies could be performed. These would include the use of pre- and post-vaccination sera as vaccine responses were found to vary between individuals, and low vaccine responses observed particular where high IgG titres were determined in pre-vaccination samples.

Correlation between IgG titres determined using the liquichip assay and ELISA would need to be performed, as this is the accepted method used for the measurement of vaccine induced IgG responses. Additional correlation between the Liquichip and SBA would need to be established.

In conclusion, a meningococcal PorA multiplex immunoassay described in this thesis was found to be a rapid, specific and reproducible method in which IgG responses to seven serosubtypes of PorA can be determined. These responses can be determined simultaneously with the requirement for only a very small volume of sera. Advantages of small sera volumes, high throughput of sera and simplicity of this assay, make the PorA multiplex assay a viable alternative to the standard ELISA. The ease with which additional serosubtypes and novel antigens can be introduced into the multiplex will help to speed up in determining the complete immune response to vaccination or carriage of meningococcal isolates. The optimal study population of and vaccine for the Liquichip assay described in this thesis, would be infant sera following immunisation with a multivalent PorA vaccine, such as Hexamen and Nonamen. The ultimate goal would be the use of an extended assay used for the evaluation of responses to a multi-component vaccine.

1. Aaberge, I. S., Oster, P., Helland, O. S., Kristoffersen, A. C., Ypma, E., Hoiby, E. A., Feiring, B., & Nokleby, H. 2005, "Combined administration of meningococcal serogroup B outer membrane vesicle vaccine and conjugated serogroup C vaccine indicated for prevention of meningococcal disease is safe and immunogenic", *Clin.Diagn.Lab Immunol.,* vol. 12, no. 5, pp. 599-605

2. Abdillahi, H. & Poolman, J. T. 1988, "Neisseria meningitidis group B serosubtyping using monoclonal antibodies in whole-cell ELISA", *Microb.Pathog.,* vol. 4, no. 1, pp. 27-32

3. Abdillahi, H. & Poolman, J. T. 1988, "Typing of group-B Neisseria meningitidis with monoclonal antibodies in the whole-cell ELISA", *J.Med.Microbiol.,* vol. 26, no. 3, pp. 177-180

4. Achtman, M. 1995, "Epidemic spread and antigenic variability of Neisseria meningitidis", *Trends Microbiol.,* vol. 3, no. 5, pp. 186-192

5. Achtman, M. 2008, "Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens", *Annu.Rev.Microbiol.,* vol. 62, pp. 53-70

6. Achtman, M., van der Ende, A., Zhu, P., Koroleva, I. S., Kusecek, B., Morelli, G., Schuurman, I. G., Brieske, N., Zurth, K., Kostyukova, N. N., & Platonov, A. E. 2001, "Molecular epidemiology of serogroup a meningitis in Moscow, 1969 to 1997", *Emerg.Infect.Dis.,* vol. 7, no. 3, pp. 420-427

7. Aguilera, J. F., Perrocheau, A., Meffre, C., & Hahne, S. 2002, "Outbreak of serogroup W135 meningococcal disease after the Hajj pilgrimage, Europe, 2000", *Emerg.Infect.Dis.,* vol. 8, no. 8, pp. 761-767

8. Ala'Aldeen, D. A., Neal, K. R., Ait-Tahar, K., Nguyen-Van-Tam, J. S., English, A., Falla, T. J., Hawkey, P. M., & Slack, R. C. 2000, "Dynamics of meningococcal long-term carriage among university students and their implications for mass vaccination", *J.Clin.Microbiol.,* vol. 38, no. 6, pp. 2311-2316

9. Alcala, B., Salcedo, C., Arreaza, L., Abad, R., Enriquez, R., De La, F. L., Uria, M. J., & Vazquez, J. A. 2004, "Antigenic and/or phase variation of PorA protein in non-subtypable Neisseria meningitidis strains isolated in Spain", *J.Med.Microbiol.,* vol. 53, no. Pt 6, pp. 515-518

10. Anderson, E. L., Bowers, T., Mink, C. M., Kennedy, D. J., Belshe, R. B., Harakeh, H., Pais, L., Holder, P., & Carlone, G. M. 1994, "Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults", *Infect.Immun.,* vol. 62, no. 8, pp. 3391-3395

11. Antharavally, B. S. 2012, "Removal of detergents from proteins and peptides in a spin-column format", *Curr.Protoc.Protein Sci.,* vol. Chapter 6, pp. Unit

12. Arachea, B. T., Sun, Z., Potente, N., Malik, R., Isailovic, D., & Viola, R. E. 2012, "Detergent selection for enhanced extraction of membrane proteins", *Protein Expr.Purif.,* vol. 86, no. 1, pp. 12-20

13. Arhin, F. F., Moreau, F., Coulton, J. W., & Mills, E. L. 1997, "Outer membrane proteins and serosubtyping with outer membrane vesicles from clinical isolates of Neisseria meningitidis", *Curr.Microbiol.,* vol. 34, no. 1, pp. 18-22

14. Auckland, C., Gray, S., Borrow, R., Andrews, N., Goldblatt, D., Ramsay, M., & Miller, E. 2006, "Clinical and immunologic risk factors for meningococcal C conjugate vaccine failure in the United Kingdom", *J.Infect.Dis.,* vol. 194, no. 12, pp. 1745-1752

15. Austen, K. F. & Fearon, D. T. 1979, "A molecular basis of activation of the alternative pathway of human complement", *Adv.Exp.Med.Biol.,* vol. 120B, pp. 3-17

16. Baker, M. G., Martin, D. R., Kieft, C. E., & Lennon, D. 2001, "A 10-year serogroup B meningococcal disease epidemic in New Zealand: descriptive epidemiology, 1991-2000", *J.Paediatr.Child Health,* vol. 37, no. 5, pp. S13-S19

17. Bakke, H., Lie, K., Haugen, I. L., Korsvold, G. E., Hoiby, E. A., Naess, L. M., Holst, J., Aaberge, I. S., Oftung, F., & Haneberg, B. 2001, "Meningococcal outer membrane vesicle vaccine given intranasally can induce immunological memory and booster responses without evidence of tolerance", *Infect.Immun.,* vol. 69, no. 8, pp. 5010-5015

18. Balmer, P., Borrow, R., & Miller, E. 2002, "Impact of meningococcal C conjugate vaccine in the UK", *J.Med.Microbiol.,* vol. 51, no. 9, pp. 717-722

19. Bambini, S., Muzzi, A., Olcen, P., Rappuoli, R., Pizza, M., & Comanducci, M. 2009, "Distribution and genetic variability of three vaccine components in a panel of strains representative of the diversity of serogroup B meningococcus", *Vaccine,* vol. 27, no. 21, pp. 2794-2803

20. Beernink, P. T., Welsch, J. A., Harrison, L. H., Leipus, A., Kaplan, S. L., & Granoff, D. M. 2007, "Prevalence of factor H-binding protein variants and NadA among meningococcal group B isolates from the United States: implications for the development of a multicomponent group B vaccine", *J.Infect.Dis.,* vol. 195, no. 10, pp. 1472-1479

21. Belo, E. F., Farhat, C. K., & Gaspari, E. N. 2010, "Comparison of dot-ELISA and standard ELISA for detection of Neisseria meningitidis outer membrane complex-specific antibodies", *Braz.J.Infect.Dis.,* vol. 14, no. 1, pp. 35-40

22. Bernardini, G., Braconi, D., Martelli, P., & Santucci, A. 2007, "Postgenomics of Neisseria meningitidis for vaccines development", *Expert.Rev.Proteomics.,* vol. 4, no. 5, pp. 667-677

23. Bettinger, J. A., Scheifele, D. W., Halperin, S. A., Vaudry, W., Findlow, J., Borrow, R., Medini, D., & Tsang, R. 2013, "Diversity of Canadian meningococcal serogroup B isolates and estimated coverage by an investigational meningococcal serogroup B vaccine (4CMenB)", *Vaccine,*

24. Beveridge, T. J. 1999, "Structures of gram-negative cell walls and their derived membrane vesicles", *The Journal of Bacteriology,* vol. 181, no. 16, pp. 4725-4733

25. Bidmos, F. A., Neal, K. R., Oldfield, N. J., Turner, D. P., Ala'Aldeen, D. A., & Bayliss, C. D. 2011, "Persistence, replacement, and rapid clonal expansion of meningococcal carriage isolates in a 2008 university student cohort", *J.Clin.Microbiol.,* vol. 49, no. 2, pp. 506-512

26. Bilukha, O. O. & Rosenstein, N. 2005, "Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP)", *MMWR Recomm.Rep.,* vol. 54, no. RR-7, pp. 1-21

27. Bjune, G., Hoiby, E. A., Gronnesby, J. K., Arnesen, O., Fredriksen, J. H., Halstensen, A., Holten, E., Lindbak, A. K., Nokleby, H., & Rosenqvist, E. 1991, "Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway", *Lancet,* vol. 338, no. 8775, pp. 1093-1096

28. Boisier, P., Nicolas, P., Djibo, S., Taha, M. K., Jeanne, I., Mainassara, H. B., Tenebray, B., Kairo, K. K., Giorgini, D., & Chanteau, S. 2007, "Meningococcal meningitis: unprecedented incidence of serogroup X-related cases in 2006 in Niger", *Clin.Infect.Dis.,* vol. 44, no. 5, pp. 657-663

29. Booy, R., Habibi, P., Nadel, S., de, M. C., Britto, J., Morrison, A., & Levin, M. 2001, "Reduction in case fatality rate from meningococcal disease associated with improved healthcare delivery", *Arch.Dis.Child,* vol. 85, no. 5, pp. 386-390

30. Borgers, H., Moens, L., Picard, C., Jeurissen, A., Raes, M., Sauer, K., Proesmans, M., De, B. K., Casanova, J. L., Meyts, I., & Bossuyt, X. 2010, "Laboratory diagnosis of specific antibody deficiency to pneumococcal capsular polysaccharide antigens by multiplexed bead assay", *Clin.Immunol.,* vol. 134, no. 2, pp. 198-205

31. Borrow, R., Andrews, N., Findlow, H., Waight, P., Southern, J., Crowley-Luke, A., Stapley, L., England, A., Findlow, J., & Miller, E. 2010, "Kinetics of antibody persistence following administration of a combination meningococcal serogroup C and haemophilus influenzae type b conjugate vaccine in healthy infants in the United Kingdom primed with a monovalent meningococcal serogroup C vaccine", *Clin.Vaccine Immunol.,* vol. 17, no. 1, pp. 154-159

32. Borrow, R., Carlone, G. M., Rosenstein, N., Blake, M., Feavers, I., Martin, D., Zollinger, W., Robbins, J., Aaberge, I., Granoff, D. M., Miller, E., Plikaytis, B., van, A. L., Poolman, J., Rappuoli, R., Danzig, L., Hackell, J., Danve, B., Caulfield, M., Lambert, S., & Stephens, D. 2006, "Neisseria meningitidis group B correlates of protection and assay standardization--international meeting report Emory University, Atlanta, Georgia, United States, 16-17 March 2005", *Vaccine,* vol. 24, no. 24, pp. 5093-5107

33. Borrow, R. & Miller, E. 2006, "Long-term protection in children with meningococcal C conjugate vaccination: lessons learned", *Expert.Rev.Vaccines.,* vol. 5, no. 6, pp. 851-857

34. Boutriau, D., Poolman, J., Borrow, R., Findlow, J., Domingo, J. D., Puig-Barbera, J., Baldo, J. M., Planelles, V., Jubert, A., Colomer, J., Gil, A., Levie, K., Kervyn, A. D., Weynants, V., Dominguez, F., Barbera, R., & Sotolongo, F. 2007, "Immunogenicity and safety of three doses of a bivalent (B:4:p1.19,15 and B:4:p1.7-2,4) meningococcal outer membrane vesicle vaccine in healthy adolescents", *Clin.Vaccine Immunol.,* vol. 14, no. 1, pp. 65-73

35. Broome, C. V. 1986, "The carrier state: Neisseria meningitidis", *J.Antimicrob.Chemother.,* vol. 18 Suppl A, pp. 25-34

36. Bruce, M. G., Rosenstein, N. E., Capparella, J. M., Shutt, K. A., Perkins, B. A., & Collins, M. 2001, "Risk factors for meningococcal disease in college students", *JAMA,* vol. 286, no. 6, pp. 688-693

37. Bullen, J. J., Rogers, H. J., & Griffiths, E. 1978, "Role of iron in bacterial infection", *Curr.Top.Microbiol.Immunol.,* vol. 80, pp. 1-35

38. Campbell, H., Andrews, N., Borrow, R., Trotter, C., & Miller, E. 2010, "Updated postlicensure surveillance of the meningococcal C conjugate vaccine in England and Wales: effectiveness, validation of serological correlates of protection, and modeling predictions of the duration of herd immunity", *Clin.Vaccine Immunol.,* vol. 17, no. 5, pp. 840-847

39. Campbell, H., Borrow, R., Salisbury, D., & Miller, E. 2009, "Meningococcal C conjugate vaccine: the experience in England and Wales", *Vaccine,* vol. 27 Suppl 2, pp. B20-B29

40. Carlone, G. M., Frasch, C. E., Siber, G. R., Quataert, S., Gheesling, L. L., Turner, S. H., Plikaytis, B. D., Helsel, L. O., DeWitt, W. E., Bibb, W. F., & . 1992, "Multicenter comparison of levels of antibody to the Neisseria meningitidis group A capsular polysaccharide measured by using an enzyme-linked immunosorbent assay", *J.Clin.Microbiol.,* vol. 30, no. 1, pp. 154-159

41. Carson, R. T. & Vignali, D. A. 1999, "Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay", *J.Immunol.Methods,* vol. 227, no. 1-2, pp. 41-52

42. Cartwright, K., Morris, R., Rumke, H., Fox, A., Borrow, R., Begg, N., Richmond, P., & Poolman, J. 1999, "Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins", *Vaccine,* vol. 17, no. 20-21, pp. 2612-2619

43. Cartwright, K. A., Stuart, J. M., Jones, D. M., & Noah, N. D. 1987, "The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica", *Epidemiol.Infect.,* vol. 99, no. 3, pp. 591-601

44. Caugant, D. A., Bovre, K., Gaustad, P., Bryn, K., Holten, E., Hoiby, E. A., & Froholm, L. O. 1986, "Multilocus genotypes determined by enzyme electrophoresis of Neisseria meningitidis isolated from patients with systemic disease and from healthy carriers", *J.Gen.Microbiol.,* vol. 132, no. 3, pp. 641-652

45. Caugant, D. A., Froholm, L. O., Bovre, K., Holten, E., Frasch, C. E., Mocca, L. F., Zollinger, W. D., & Selander, R. K. 1986, "Intercontinental spread of a genetically distinctive complex of clones of Neisseria meningitidis causing epidemic disease", *Proc.Natl.Acad.Sci.U.S.A,* vol. 83, no. 13, pp. 4927-4931

46. Caugant, D. A., Hoiby, E. A., Magnus, P., Scheel, O., Hoel, T., Bjune, G., Wedege, E., Eng, J., & Froholm, L. O. 1994, "Asymptomatic carriage of Neisseria meningitidis in a randomly sampled population", *J.Clin.Microbiol.,* vol. 32, no. 2, pp. 323-330

47. Caugant, D. A., Mocca, L. F., Frasch, C. E., Froholm, L. O., Zollinger, W. D., & Selander, R. K. 1987, "Genetic structure of Neisseria meningitidis populations in relation to serogroup, serotype, and outer membrane protein pattern", *The Journal of Bacteriology,* vol. 169, no. 6, pp. 2781-2792

48. Chatt, C., Gajraj, R., Hawker, J., Neal, K., Tahir, M., Lawrence, M., Gray, S. J., Lucidarme, J., Carr, A. D., Clark, S. A., & Fowler.T 2014, "Four-month outbreak of Invasive Meningococcal disease caused by a rare serogroup B strain, identified through the use of molecular PorA subtyping, England, 2013", *Euro.Surveill,* vol. 19, no. 44,

49. Christensen, H., May, M., Bowen, L., Hickman, M., & Trotter, C. L. 2010, "Meningococcal carriage by age: a systematic review and meta-analysis", *Lancet Infect.Dis.,* vol. 10, no. 12, pp. 853-861

50. Claassen, I., Meylis, J., van der Ley, P., Peeters, C., Brons, H., Robert, J., Borsboom, D., van der Ark, A., van Straaten, I., Roholl, P., Kuipers, B., & Poolman, J. 1996, "Production, characterization and control of a Neisseria meningitidis hexavalent class 1 outer membrane protein containing vesicle vaccine", *Vaccine,* vol. 14, no. 10, pp. 1001-1008

51. Clarke, S. C., Diggle, M. A., Molling, P., Unemo, M., & Olcen, P. 2003, "Analysis of PorA variable region 3 in meningococci: implications for vaccine policy?", *Vaccine,* vol. 21, no. 19-20, pp. 2468-2473

52. Cochi, S. L., Markowitz, L. E., Joshi, D. D., Owens, R. C., Jr., Stenhouse, D. H., Regmi, D. N., Shrestha, R. P., Lacharya, I., Manandhar, M., Gurubacharya, V. L., & . 1987, "Control of epidemic group A meningococcal meningitis in Nepal", *Int.J.Epidemiol.,* vol. 16, no. 1, pp. 91-97

53. Codorean, E., Nichita, C., Albulescu, L., Raducan, E., Popescu, I. D., Lonita, A. C., & Albulescu, R. 2010, "Correlation of XMAP and ELISA cytokine profiles; development and validation for immunotoxicological studies in vitro", *Roum.Arch.Microbiol.Immunol.,* vol. 69, no. 1, pp. 13-19

54. Coll, P., Borche, L., Ausina, V., Mirelis, B., & Prats, G. 1986, "Dot-immunobinding assay with a monoclonal antibody for detection of group B meningococcal antigen", *Eur.J.Clin.Microbiol.,* vol. 5, no. 1, pp. 44-46

55. Collins, B. S. 2011, "Gram-negative outer membrane vesicles in vaccine development", *Discov.Med.,* vol. 12, no. 62, pp. 7-15

56. Crowe, J., Masone, B. S., & Ribbe, J. 1995, "One-step purification of recombinant proteins with the 6xHis tag and Ni-NTA resin", *Mol.Biotechnol.,* vol. 4, no. 3, pp. 247-258

57. Dai, Z., Hu, S., Chen, M., Lu, C., Wang, Y., Liu, Q., Zhang, Y., Yu, M., Zhu, C., & Li, Z. 2010, "Construction, expression and immunocompetence of Neisseria gonorrhoeae porB and Escherichia coli itB fusion gene", *Wei Sheng Wu Xue.Bao.,* vol. 50, no. 4, pp. 517-523

58. Danzig, L. 2004, "Meningococcal vaccines", *Pediatr.Infect.Dis.J.,* vol. 23, no. 12 Suppl, pp. S285-S292

59. Davenport, V., Guthrie, T., Findlow, J., Borrow, R., Williams, N. A., & Heyderman, R. S. 2003, "Evidence for naturally acquired T cell-mediated mucosal immunity to Neisseria meningitidis", *J.Immunol.,* vol. 171, no. 8, pp. 4263-4270

60. de Filippis, I. 2009, "Quest for a broad-range vaccine against Neisseria meningitidis serogroup B: implications of genetic variations of the surface-exposed proteins", *J.Med.Microbiol.,* vol. 58, no. Pt 9, pp. 1127-1132

61. de Filippis, I., de Andrade, C. F., Silva, L., Prevots, D. R., & Vicente, A. C. 2007, "PorA variable antigenic regions VR1, VR2, and VR3 of Neisseria meningitidis serogroups B and C isolated in Brazil from 1999 to 2004", *Infect.Immun.,* vol. 75, no. 7, pp. 3683-3685

62. de Filippis, I., Gopalan, V., & Huyen, Y. 2011, "PorA VR3 Typing Database: a web-based resource for the determination of PorA VR3 alleles of Neisseria meningitidis", *Infect.Genet.Evol.,* vol. 11, no. 1, pp. 248-249

63. de Kleijn, E., van Eijndhoven, L., Vermont, C., Kuipers, B., van Dijken, H., Rumke, H., de Groot, R., van Alphen, L., & van den Dobbelsteen, G. 2001, "Serum bactericidal activity and isotype distribution of antibodies in toddlers and schoolchildren after vaccination with RIVM hexavalent PorA vesicle vaccine", *Vaccine,* vol. 20, no. 3-4, pp. 352-358

64. de Kleijn, E. D., de, G. R., Labadie, J., Lafeber, A. B., van den, D. G., van, A. L., van, D. H., Kuipers, B., van Omme, G. W., Wala, M., Juttmann, R., & Rumke, H. C. 2000, "Immunogenicity and safety of a hexavalent meningococcal outer-membrane-vesicle vaccine in children of 2-3 and 7-8 years of age", *Vaccine,* vol. 18, no. 15, pp. 1456-1466

65. de Moraes, J. C., Perkins, B. A., Camargo, M. C., Hidalgo, N. T., Barbosa, H. A., Sacchi, C. T., Landgraf, I. M., Gattas, V. L., Vasconcelos, H. G., & . 1992, "Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil", *Lancet,* vol. 340, no. 8827, pp. 1074-1078

66. de Voer, R. M., Schepp, R. M., Versteegh, F. G., van der Klis, F. R., & Berbers, G. A. 2009, "Simultaneous detection of Haemophilus influenzae type b polysaccharide-specific antibodies and Neisseria meningitidis serogroup A, C, Y, and W-135 polysaccharide-specific antibodies in a fluorescent-bead-based multiplex immunoassay", *Clin.Vaccine Immunol.,* vol. 16, pp. 433-436

67. de Voer, R. M., van der Klis, F. R., Engels, C. W., Rijkers, G. T., Sanders, E. A., & Berbers, G. A. 2008, "Development of a fluorescent-bead-based multiplex immunoassay to determine immunoglobulin G subclass responses to Neisseria meningitidis serogroup A and C polysaccharides", *Clin.Vaccine Immunol.,* vol. 15, no. 8, pp. 1188-1193

68. de Vries, F. P., Cole, R., Dankert, J., Frosch, M., & van Putten, J. P. 1998, "Neisseria meningitidis producing the Opc adhesin binds epithelial cell proteoglycan receptors", *Mol.Microbiol.,* vol. 27, no. 6, pp. 1203-1212

69. Decosas, J. & Koama, J. B. 2002, "Chronicle of an outbreak foretold: meningococcal meningitis W135 in Burkina Faso", *Lancet Infect.Dis.,* vol. 2, no. 12, pp. 763-765

70. Delbos, V., Lemee, L., Benichou, J., Berthelot, G., Deghmane, A. E., Leroy, J. P., Houivet, E., Hong, E., Taha, M. K., & Caron, F. 2013, "Impact of MenBvac, an outer membrane vesicle (OMV) vaccine, on the meningococcal carriage", *Vaccine,* vol. 31, no. 40, pp. 4416-4420

71. Delisle, E., Larrieu, S., Simoes, J., Laylle, N., De, P. M., Taha, M. K., Termignon, J. L., & Parent, d. C., I 2010, "Community outbreak of group B meningococcal disease in southwest France--December 2008 to September 2009", *Euro.Surveill,* vol. 15, no. 37,

72. Delrieu, I., Yaro, S., Tamekloe, T. A., Njanpop-Lafourcade, B. M., Tall, H., Jaillard, P., Ouedraogo, M. S., Badziklou, K., Sanou, O., Drabo, A., Gessner, B. D., Kambou, J. L., & Mueller, J. E. 2011, "Emergence of epidemic Neisseria meningitidis serogroup X meningitis in Togo and Burkina Faso", *PLoS.One.,* vol. 6, no. 5, pp. e19513

73. Delvig, A. A., Michaelsen, T. E., Aase, A., Hoiby, E. A., & Rosenqvist, E. 1997, "Vaccine-induced IgG antibodies to the linear epitope on the PorB outer membrane protein promote opsonophagocytosis of Neisseria meningitidis by human neutrophils", *Clin.Immunol.Immunopathol.,* vol. 84, no. 1, pp. 27-35

74. Derrick, J. P., Urwin, R., Suker, J., Feavers, I. M., & Maiden, M. C. 1999, "Structural and evolutionary inference from molecular variation in Neisseria porins", *Infect.Immun.,* vol. 67, no. 5, pp. 2406-2413

75. Didelot, X., Urwin, R., Maiden, M. C., & Falush, D. 2009, "Genealogical typing of Neisseria meningitidis", *Microbiology,* vol. 155, no. Pt 10, pp. 3176-3186

76. Djingarey, M. H., Barry, R., Bonkoungou, M., Tiendrebeogo, S., Sebgo, R., Kandolo, D., Lingani, C., Preziosi, M. P., Zuber, P. L., Perea, W., Hugonnet, S., lepiane de Rey, T. N., Tevi-Benissan, C., Clark, T. A., Mayer, L. W., Novak, R., Messonier, N. E., Berlier, M., Toboe, D., Nshimirimana, D., Mihigo, R., Aguado, T., Diomande, F., Kristiansen, P. A., Caugant, D. A., & Laforce, F. M. 2012, "Effectively introducing a new meningococcal A conjugate vaccine in Africa: the Burkina Faso experience", *Vaccine,* vol. 30 Suppl 2, pp. B40-B45

77. Donnelly, J., Medini, D., Boccadifuoco, G., Biolchi, A., Ward, J., Frasch, C., Moxon, E. R., Stella, M., Comanducci, M., Bambini, S., Muzzi, A., Andrews, W., Chen, J., Santos, G., Santini, L., Boucher, P., Serruto, D., Pizza, M., Rappuoli, R., & Giuliani, M. M. 2010, "Qualitative and quantitative assessment of meningococcal antigens to evaluate the potential strain coverage of protein-based vaccines", *Proc.Natl.Acad.Sci.U.S.A,* vol. 107, no. 45, pp. 19490-19495

78. Dunbar, S. A., Vander Zee, C. A., Oliver, K. G., Karem, K. L., & Jacobson, J. W. 2003, "Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system", *J Microbiol.Methods,* vol. 53, pp. 245-252

79. Dyet, K. H. & Martin, D. R. 2005, "Sequence variation in the porB gene from B:P1.4 meningococci causing New Zealand's epidemic", *J.Clin.Microbiol.,* vol. 43, no. 2, pp. 838-842

80. Dyet, K. H., Simmonds, R. S., & Martin, D. R. 2004, "Evaluation of porB PCR-amplicon restriction endonuclease analysis as a method to determine porB variable-region sequences in nonserotypeable meningococci", *J.Clin.Microbiol.,* vol. 42, no. 4, pp. 1731-1733

81. Edmond, K., Clark, A., Korczak, V. S., Sanderson, C., Griffiths, U. K., & Rudan, I. 2010, "Global and regional risk of disabling sequelae from bacterial meningitis: a systematic review and meta-analysis", *Lancet Infect.Dis.,* vol. 10, no. 5, pp. 317-328

82. Erlich, K. S. & Congeni, B. L. 2012, "Importance of circulating antibodies in protection against meningococcal disease", *Hum.Vaccin.Immunother.,* vol. 8, no. 8, pp. 1029-1035

83. Farnaud, S. & Evans, R. W. 2003, "Lactoferrin--a multifunctional protein with antimicrobial properties", *Mol.Immunol.,* vol. 40, no. 7, pp. 395-405

84. Feavers, I. M., Gray, S. J., Urwin, R., Russell, J. E., Bygraves, J. A., Kaczmarski, E. B., & Maiden, M. C. 1999, "Multilocus sequence typing and antigen gene sequencing in

the investigation of a meningococcal disease outbreak", *J.Clin.Microbiol.,* vol. 37, no. 12, pp. 3883-3887

85. Feavers, I. M., Suker, J., McKenna, A. J., Heath, A. B., & Maiden, M. C. 1992, "Molecular analysis of the serotyping antigens of Neisseria meningitidis", *Infect.Immun.,* vol. 60, no. 9, pp. 3620-3629

86. Feiring, B., Fuglesang, J., Oster, P., Naess, L. M., Helland, O. S., Tilman, S., Rosenqvist, E., Bergsaker, M. A., Nokleby, H., & Aaberge, I. S. 2006, "Persisting immune responses indicating long-term protection after booster dose with meningococcal group B outer membrane vesicle vaccine", *Clin.Vaccine Immunol.,* vol. 13, no. 7, pp. 790-796

87. Ferguson, L. E., Hormann, M. D., Parks, D. K., & Yetman, R. J. 2002, "Neisseria meningitidis: presentation, treatment, and prevention", *J.Pediatr.Health Care,* vol. 16, no. 3, pp. 119-124

88. Ferrari, G., Garaguso, I., du-Bobie, J., Doro, F., Taddei, A. R., Biolchi, A., Brunelli, B., Giuliani, M. M., Pizza, M., Norais, N., & Grandi, G. 2006, "Outer membrane vesicles from group B Neisseria meningitidis delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles", *Proteomics,* vol. 6, no. 6, pp. 1856-1866

89. Findlow, H., Borrow, R., Andrews, N., Waight, P., Sheasby, E., Matheson, M., England, A., Goldblatt, D., Ashton, L., Findlow, J., & Miller, E. 2012, "Immunogenicity of a single dose of meningococcal group C conjugate vaccine given at 3 months of age to healthy infants in the United kingdom", *Pediatr.Infect.Dis.J.,* vol. 31, no. 6, pp. 616-622

90. Findlow, J., Borrow, R., Snape, M. D., Dawson, T., Holland, A., John, T. M., Evans, A., Telford, K. L., Ypma, E., Toneatto, D., Oster, P., Miller, E., & Pollard, A. J. 2010, "Multicenter, open-label, randomized phase II controlled trial of an investigational recombinant Meningococcal serogroup B vaccine with and without outer membrane vesicles, administered in infancy", *Clin.Infect.Dis.,* vol. 51, no. 10, pp. 1127-1137

91. Findlow, J., Lowe, A., Deane, S., Balmer, P., van den, D. G., Dawson, M., Andrews, N., & Borrow, R. 2005, "Effect of sequence variation in meningococcal PorA outer membrane protein on the effectiveness of a hexavalent PorA outer membrane vesicle vaccine in toddlers and school children", *Vaccine,* vol. 23, no. 20, pp. 2623-2627

92. Findlow, J., Taylor, S., Aase, A., Horton, R., Heyderman, R., Southern, J., Andrews, N., Barchha, R., Harrison, E., Lowe, A., Boxer, E., Heaton, C., Balmer, P., Kaczmarski, E., Oster, P., Gorringe, A., Borrow, R., & Miller, E. 2006, "Comparison and correlation of neisseria meningitidis serogroup B immunologic assay results and human antibody responses following three doses of the Norwegian meningococcal outer membrane vesicle vaccine MenBvac", *Infect.Immun.,* vol. 74, no. 8, pp. 4557-4565

93. Finne, J., Leinonen, M., & Makela, P. H. 1983, "Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis", *Lancet,* vol. 2, no. 8346, pp. 355-357

94. Fitzgerald, C., Collins, M., van, D. S., Mikoleit, M., Brown, T., & Fields, P. 2007, "Multiplex, bead-based suspension array for molecular determination of common Salmonella serogroups", *J Clin.Microbiol.,* vol. 45, pp. 3323-3334

95. Fletcher, L. D., Bernfield, L., Barniak, V., Farley, J. E., Howell, A., Knauf, M., Ooi, P., Smith, R. P., Weise, P., Wetherell, M., Xie, X., Zagursky, R., Zhang, Y., & Zlotnick, G. W. 2004, "Vaccine potential of the Neisseria meningitidis 2086 lipoprotein", *Infect.Immun.,* vol. 72, no. 4, pp. 2088-2100

96. Fonseca, B. P., Marques, C. F., Nascimento, L. D., Mello, M. B., Silva, L. B., Rubim, N. M., Foti, L., Silva, E. D., Ferreira, A. G., & Krieger, M. A. 2011, "Development of a multiplex bead-based assay for detection of hepatitis C virus", *Clin.Vaccine Immunol.,* vol. 18, no. 5, pp. 802-806

97. Frasch, C. E. 1989, "Vaccines for prevention of meningococcal disease", *Clin.Microbiol.Rev.,* vol. 2 Suppl, pp. S134-S138

98. Frasch, C. E. & Chapman, S. S. 1972, "Classification of Neisseria meningitidis group B into distinct serotypes. I. Serological typing by a microbactericidal method", *Infect.Immun.,* vol. 5, no. 1, pp. 98-102

99. Frasch, C. E., Tsai, C. M., & Mocca, L. F. 1986, "Outer membrane proteins of Neisseria meningitidis: structure and importance in meningococcal disease", *Clin.Invest Med.,* vol. 9, no. 2, pp. 101-107

100. Frasch, C. E., van, A. L., Holst, J., Poolman, J. T., & Rosenqvist, E. 2001, "Outer membrane protein vesicle vaccines for meningococcal disease", *Methods Mol.Med.,* vol. 66, pp. 81-107

101. Frasch, C. E., Zollinger, W. D., & Poolman, J. T. 1985, "Serotype antigens of Neisseria meningitidis and a proposed scheme for designation of serotypes", *Rev.Infect.Dis.,* vol. 7, no. 4, pp. 504-510

102. Fredriksen, J. H., Rosenqvist, E., Wedege, E., Bryn, K., Bjune, G., Froholm, L. O., Lindbak, A. K., Mogster, B., Namork, E., Rye, U., & . 1991, "Production, characterization and control of MenB-vaccine "Folkehelsa": an outer membrane vesicle vaccine against group B meningococcal disease", *NIPH Ann.,* vol. 14, no. 2, pp. 67-79

103. Fulton, R. J., McDade, R. L., Smith, P. L., Kienker, L. J., & Kettman, J. R., Jr. 1997, "Advanced multiplexed analysis with the FlowMetrix system", *Clin.Chem.,* vol. 43, no. 9, pp. 1749-1756

104. Galloway, Y., Stehr-Green, P., McNicholas, A., & O'Hallahan, J. 2009, "Use of an observational cohort study to estimate the effectiveness of the New Zealand group B meningococcal vaccine in children aged under 5 years", *Int.J.Epidemiol.,* vol. 38, no. 2, pp. 413-418

105. Gasparini, R. & Panatto, D. 2011, "Meningococcal glycoconjugate vaccines", *Hum.Vaccin.,* vol. 7, no. 2,

106. Gheesling, L. L., Carlone, G. M., Pais, L. B., Holder, P. F., Maslanka, S. E., Plikaytis, B. D., Achtman, M., Densen, P., Frasch, C. E., Kayhty, H., & . 1994, "Multicenter comparison of Neisseria meningitidis serogroup C anti-capsular polysaccharide antibody levels measured by a standardized enzyme-linked immunosorbent assay", *J.Clin.Microbiol.,* vol. 32, no. 6, pp. 1475-1482

107. Girard, M. P., Preziosi, M. P., Aguado, M. T., & Kieny, M. P. 2006, "A review of vaccine research and development: meningococcal disease", *Vaccine,* vol. 24, no. 22, pp. 4692-4700

108. Giuliani, M. M., du-Bobie, J., Comanducci, M., Arico, B., Savino, S., Santini, L., Brunelli, B., Bambini, S., Biolchi, A., Capecchi, B., Cartocci, E., Ciucchi, L., Di, M. F., Ferlicca, F., Galli, B., Luzzi, E., Masignani, V., Serruto, D., Veggi, D., Contorni, M., Morandi, M., Bartalesi, A., Cinotti, V., Mannucci, D., Titta, F., Ovidi, E., Welsch, J. A., Granoff, D., Rappuoli, R., & Pizza, M. 2006, "A universal vaccine for serogroup B meningococcus", *Proc.Natl.Acad.Sci.U.S.A,* vol. 103, no. 29, pp. 10834-10839

109. Gold, R., Goldschneider, I., Lepow, M. L., Draper, T. F., & Randolph, M. 1978, "Carriage of Neisseria meningitidis and Neisseria lactamica in infants and children", *J.Infect.Dis.,* vol. 137, no. 2, pp. 112-121

110. Gold, R., Lepow, M. L., Goldschneider, I., Draper, T. F., & Gotschlich, E. C. 1978, "Antibody responses of human infants to three doses of group A Neisseria meningitidis polysaccharide vaccine administered at two, four, and six months of age", *J.Infect.Dis.,* vol. 138, no. 6, pp. 731-735

111. Gold, R., Lepow, M. L., Goldschneider, I., Draper, T. L., & Gotschlich, E. C. 1975, "Clinical evaluation of group A and group C meningococcal polysaccharide vaccines in infants", *J.Clin.Invest,* vol. 56, no. 6, pp. 1536-1547

112. Goldblatt, D. 2000, "Conjugate vaccines", *Clin.Exp.Immunol.,* vol. 119, no. 1, pp. 1-3

113. Goldschneider, I., Gotschlich, E. C., & Artenstein, M. S. 1969, "Human immunity to the meningococcus. I. The role of humoral antibodies", *J.Exp.Med.,* vol. 129, no. 6, pp. 1307-1326

114. Goldschneider, I., Gotschlich, E. C., & Artenstein, M. S. 1969, "Human immunity to the meningococcus. II. Development of natural immunity", *J.Exp.Med.,* vol. 129, no. 6, pp. 1327-1348

115. Gorla, M. C., Lemos, A. P., Sacchi, C. T., de Moraes, J. C., & Milagres, L. G. 2003, "Comparison of PorA VR types and porA promoter sequence from Neisseria meningitidis B isolated from non-immunised children and vaccine failures immunised with a serogroup B outer membrane protein vaccine", *Vaccine,* vol. 21, no. 21-22, pp. 2871-2876

116. Gorringe, A. R., Taylor, S., Brookes, C., Matheson, M., Finney, M., Kerr, M., Hudson, M., Findlow, J., Borrow, R., Andrews, N., Kafatos, G., Evans, C. M., & Read, R. C. 2009, "Phase I safety and immunogenicity study of a candidate meningococcal disease vaccine based on Neisseria lactamica outer membrane vesicles", *Clin.Vaccine Immunol.,* vol. 16, no. 8, pp. 1113-1120

117. Gossage, J. D. 1964, "Acute purulent Meningitis in children: Experience at the Hospital for sick children, Toronto", *Can.Med.Assoc.J.,* vol. 90, pp. 615-617

118. Gossger, N., Snape, M. D., Yu, L. M., Finn, A., Bona, G., Esposito, S., Principi, N., ez-Domingo, J., Sokal, E., Becker, B., Kieninger, D., Prymula, R., Dull, P., Ypma, E., Toneatto, D., Kimura, A., & Pollard, A. J. 2012, "Immunogenicity and tolerability of recombinant serogroup B meningococcal vaccine administered with or without routine infant vaccinations according to different immunization schedules: a randomized controlled trial", *JAMA,* vol. 307, no. 6, pp. 573-582

119. Gotschlich, E. C., Goldschneider, I., & Artenstein, M. S. 1969, "Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers", *J.Exp.Med.*, vol. 129, no. 6, pp. 1367-1384

120. Gotschlich, E. C., Goldschneider, I., & Artenstein, M. S. 1969, "Human immunity to the meningococcus. V. The effect of immunization with meningococcal group C polysaccharide on the carrier state", *J.Exp.Med.*, vol. 129, no. 6, pp. 1385-1395

121. Gotschlich, E. C., Liu, T. Y., & Artenstein, M. S. 1969, "Human immunity to the meningococcus. III. Preparation and immunochemical properties of the group A, group B, and group C meningococcal polysaccharides", *J.Exp.Med.*, vol. 129, no. 6, pp. 1349-1365

122. Goulding, J. N., Hookey, J. V., Stanley, J., Olver, W., Neal, K. R., Ala'Aldeen, D. A., & Arnold, C. 2000, "Fluorescent amplified-fragment length polymorphism genotyping of Neisseria meningitidis identifies clones associated with invasive disease", *J.Clin.Microbiol.*, vol. 38, no. 12, pp. 4580-4585

123. Granoff, D. M., Maslanka, S. E., Carlone, G. M., Plikaytis, B. D., Santos, G. F., Mokatrin, A., & Raff, H. V. 1998, "A modified enzyme-linked immunosorbent assay for measurement of antibody responses to meningococcal C polysaccharide that correlate with bactericidal responses", *Clin.Diagn.Lab Immunol.*, vol. 5, no. 4, pp. 479-485

124. Granoff, D. M. & Munson, R. S. Jr. 1986, "Prospects for prevention of Haemophilus influenzae type b disease by immunization", *J.Infect.Dis.*, vol. 153, no. 3, pp. 448-461

125. Granoff, D. M., Welsch, J. A., & Ram, S. 2009, "Binding of complement factor H (fH) to Neisseria meningitidis is specific for human fH and inhibits complement activation by rat and rabbit sera", *Infect.Immun.*, vol. 77, no. 2, pp. 764-769

126. Gray, S. J., Trotter, C. L., Ramsay, M. E., Guiver, M., Fox, A. J., Borrow, R., Mallard, R. H., & Kaczmarski, E. B. 2006, "Epidemiology of meningococcal disease in England and Wales 1993/94 to 2003/04: contribution and experiences of the Meningococcal Reference Unit", *J.Med.Microbiol.*, vol. 55, no. Pt 7, pp. 887-896

127. Greenwood, B. 1999, "Manson Lecture. Meningococcal meningitis in Africa", *Trans.R.Soc.Trop.Med.Hyg.*, vol. 93, no. 4, pp. 341-353

128. Guttormsen, H. K., Wetzler, L. M., & Solberg, C. O. 1994, "Humoral immune response to class 1 outer membrane protein during the course of meningococcal disease", *Infect.Immun.*, vol. 62, no. 4, pp. 1437-1443

129. Haghi, F., Peerayeh, S. N., Siadat, S. D., & Montajabiniat, M. 2011, "Cloning, expression and purification of outer membrane protein PorA of Neisseria meningitidis serogroup B", *J.Infect.Dev.Ctries.*, vol. 5, no. 12, pp. 856-862

130. Haj-Hassan, T. A., Thompson, M. J., Mayon-White, R. T., Ninis, N., Harnden, A., Smith, L. F., Perera, R., & Mant, D. C. 2011, "Which early 'red flag' symptoms identify children with meningococcal disease in primary care?", *Br.J.Gen.Pract.*, vol. 61, no. 584, pp. e97-104

131. Hammerschmidt, S., Hilse, R., van Putten, J. P., Gerardy-Schahn, R., Unkmeir, A., & Frosch, M. 1996, "Modulation of cell surface sialic acid expression in Neisseria

meningitidis via a transposable genetic element", *EMBO J.,* vol. 15, no. 1, pp. 192-198

132. Hammerschmidt, S., Muller, A., Sillmann, H., Muhlenhoff, M., Borrow, R., Fox, A., van, P. J., Zollinger, W. D., Gerardy-Schahn, R., & Frosch, M. 1996, "Capsule phase variation in Neisseria meningitidis serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease", *Mol.Microbiol.,* vol. 20, no. 6, pp. 1211-1220

133. Harrison, L. H. 2006, "Prospects for vaccine prevention of meningococcal infection", *Clin.Microbiol.Rev.,* vol. 19, no. 1, pp. 142-164

134. Harrison, L. H., Trotter, C. L., & Ramsay, M. E. 2009, "Global epidemiology of meningococcal disease", *Vaccine,* vol. 27 Suppl 2, pp. B51-B63

135. Hassan-King, M. K., Wall, R. A., & Greenwood, B. M. 1988, "Meningococcal carriage, meningococcal disease and vaccination", *J.Infect.,* vol. 16, no. 1, pp. 55-59

136. Hedberg, S. T., Toros, B., Fredlund, H., Olcen, P., & Molling, P. 2011, "Genetic characterisation of the emerging invasive Neisseria meningitidis serogroup Y in Sweden, 2000 to 2010", *Euro.Surveill,* vol. 16, no. 23,

137. Hill, J. C., Peterson, N. R., & Weiss, E. 1972, "Characterization of spheroplast membranes of Neisseria meningitidis group B", *Infect.Immun.,* vol. 5, no. 4, pp. 612-621

138. Hjetland, R., Caugant, D. A., Hofstad, T., Froholm, L. O., & Selander, R. K. 1990, "Serogroup A Neisseria meningitidis of clone III-1 present in western Norway as early as 1969-73", *Scand.J.Infect.Dis.,* vol. 22, no. 2, pp. 241-242

139. Holst, J., Martin, D., Arnold, R., Huergo, C. C., Oster, P., O'Hallahan, J., & Rosenqvist, E. 2009, "Properties and clinical performance of vaccines containing outer membrane vesicles from Neisseria meningitidis", *Vaccine,* vol. 27 Suppl 2, pp. B3-12

140. Humphries, H. E., Williams, J. N., Blackstone, R., Jolley, K. A., Yuen, H. M., Christodoulides, M., & Heckels, J. E. 2006, "Multivalent liposome-based vaccines containing different serosubtypes of PorA protein induce cross-protective bactericidal immune responses against Neisseria meningitidis", *Vaccine,* vol. 24, no. 1, pp. 36-44

141. Imrey, P. B., Jackson, L. A., Ludwinski, P. H., England, A. C., III, Fella, G. A., Fox, B. C., Isdale, L. B., Reeves, M. W., & Wenger, J. D. 1995, "Meningococcal carriage, alcohol consumption, and campus bar patronage in a serogroup C meningococcal disease outbreak", *J.Clin.Microbiol.,* vol. 33, no. 12, pp. 3133-3137

142. Jamba, G., Bytchenko, B., Causse, G., Cvjetanovic, B., Ocirvan, G., Tsend, N., Kupul, J., Tseren, S., Batsuri, A., Hisigdorj, A., & Burian, V. 1979, "Immunization during a cerebrospinal meningitis epidemic in the Mongolian People's Republic, 1974-75", *Bull.World Health Organ,* vol. 57, no. 6, pp. 943-946

143. Jansen, C., Wiese, A., Reubsaet, L., Dekker, N., de Cock, H., Seydel, U., & Tommassen, J. 2000, "Biochemical and biophysical characterization of in vitro folded outer membrane porin PorA of Neisseria meningitidis", *Biochim.Biophys.Acta,* vol. 1464, no. 2, pp. 284-298

144. Jarvis, G. A. 1995, "Recognition and control of neisserial infection by antibody and complement", *Trends Microbiol.,* vol. 3, no. 5, pp. 198-201

145. Jarvis, G. A. & Vedros, N. A. 1987, "Sialic acid of group B Neisseria meningitidis regulates alternative complement pathway activation", *Infect.Immun.,* vol. 55, no. 1, pp. 174-180

146. Jiang, H. Q., Hoiseth, S. K., Harris, S. L., McNeil, L. K., Zhu, D., Tan, C., Scott, A. A., Alexander, K., Mason, K., Miller, L., DaSilva, I., Mack, M., Zhao, X. J., Pride, M. W., Andrew, L., Murphy, E., Hagen, M., French, R., Arora, A., Jones, T. R., Jansen, K. U., Zlotnick, G. W., & Anderson, A. S. 2010, "Broad vaccine coverage predicted for a bivalent recombinant factor H binding protein based vaccine to prevent serogroup B meningococcal disease", *Vaccine,* vol. 28, no. 37, pp. 6086-6093

147. Jodar, L., Feavers, I. M., Salisbury, D., & Granoff, D. M. 2002, "Development of vaccines against meningococcal disease", *Lancet,* vol. 359, no. 9316, pp. 1499-1508

148. Jolley, K. A., Bliss, C. M., Bennett, J. S., Bratcher, H. B., Brehony, C., Colles, F. M., Wimalarathna, H., Harrison, O. B., Sheppard, S. K., Cody, A. J., & Maiden, M. C. 2012, "Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain", *Microbiology,* vol. 158, no. Pt 4, pp. 1005-1015

149. Jolley, K. A., Brehony, C., & Maiden, M. C. 2007, "Molecular typing of meningococci: recommendations for target choice and nomenclature", *FEMS Microbiol.Rev.,* vol. 31, no. 1, pp. 89-96

150. Jolley, K. A., Chan, M. S., & Maiden, M. C. 2004, "mlstdbNet - distributed multi-locus sequence typing (MLST) databases", *BMC.Bioinformatics.,* vol. 5, pp. 86

151. Jolley, K. A. & Maiden, M. C. 2010, "BIGSdb: Scalable analysis of bacterial genome variation at the population level", *BMC.Bioinformatics.,* vol. 11, pp. 595

152. Jones, G. R., Christodoulides, M., Brooks, J. L., Miller, A. R., Cartwright, K. A., & Heckels, J. E. 1998, "Dynamics of carriage of Neisseria meningitidis in a group of military recruits: subtype stability and specificity of the immune response following colonization", *J.Infect.Dis.,* vol. 178, no. 2, pp. 451-459

153. Jordens, J. Z., Williams, J. N., Jones, G. R., Christodoulides, M., & Heckels, J. E. 2004, "Development of immunity to serogroup B meningococci during carriage of Neisseria meningitidis in a cohort of university students", *Infect.Immun.,* vol. 72, no. 11, pp. 6503-6510

154. Kaaijk, P., van, S., I, van de Waterbeemd, B., Boot, E. P., Levels, L. M., van Dijken, H. H., & van den Dobbelsteen, G. P. 2013, "Preclinical safety and immunogenicity evaluation of a nonavalent PorA native outer membrane vesicle vaccine against serogroup B meningococcal disease", *Vaccine,* vol. 31, no. 7, pp. 1065-1071

155. Kayhty, H., Makela, P. H., & Ruoslahti, E. 1977, "Radioimmunoassay of capsular polysaccharide antigens of groups A and C meningococci and Haemophilus influenzae type b in cerebrospinal fluid", *J.Clin.Pathol.,* vol. 30, no. 9, pp. 831-833

156. Kelly, C., Arnold, R., Galloway, Y., & O'Hallahan, J. 2007, "A prospective study of the effectiveness of the New Zealand meningococcal B vaccine", *Am.J.Epidemiol.,* vol. 166, no. 7, pp. 817-823

157. Kimura, A., Toneatto, D., Kleinschmidt, A., Wang, H., & Dull, P. 2011, "Immunogenicity and safety of a multicomponent meningococcal serogroup B vaccine and a quadrivalent meningococcal CRM197 conjugate vaccine against serogroups A, C, W-135, and Y in adults who are at increased risk for occupational exposure to meningococcal isolates", *Clin.Vaccine Immunol.,* vol. 18, no. 3, pp. 483-486

158. King, W. J., MacDonald, N. E., Wells, G., Huang, J., Allen, U., Chan, F., Ferris, W., az-Mitoma, F., & Ashton, F. 1996, "Total and functional antibody response to a quadrivalent meningococcal polysaccharide vaccine among children", *J.Pediatr.,* vol. 128, no. 2, pp. 196-202

159. Kirsch, E. A., Barton, R. P., Kitchen, L., & Giroir, B. P. 1996, "Pathophysiology, treatment and outcome of meningococcemia: a review and recent experience", *Pediatr.Infect.Dis.J.,* vol. 15, no. 11, pp. 967-978

160. Koeberling, O., Giuntini, S., Seubert, A., & Granoff, D. M. 2009, "Meningococcal outer membrane vesicle vaccines derived from mutant strains engineered to express factor H binding proteins from antigenic variant groups 1 and 2", *Clin.Vaccine Immunol.,* vol. 16, no. 2, pp. 156-162

161. Kortekaas, J., Muller, S. A., Ringler, P., Gregorini, M., Weynants, V. E., Rutten, L., Bos, M. P., & Tommassen, J. 2006, "Immunogenicity and structural characterisation of an in vitro folded meningococcal siderophore receptor (FrpB, FetA)", *Microbes.Infect.,* vol. 8, no. 8, pp. 2145-2153

162. Kortekaas, J., Pettersson, A., van der Biezen, J., Weynants, V. E., van der Ley, P., Poolman, J., Bos, M. P., & Tommassen, J. 2007, "Shielding of immunogenic domains in Neisseria meningitidis FrpB (FetA) by the major variable region", *Vaccine,* vol. 25, no. 1, pp. 72-84

163. Kristiansen, B. E., Lind, K. W., Mevold, K., Sorensen, B., Froholm, L. O., Bryn, K., Tjade, T., & Bovre, K. 1988, "Meningococcal phenotypic and genotypic characteristics and human antibody levels", *J.Clin.Microbiol.,* vol. 26, no. 10, pp. 1988-1992

164. Kuespert, K., Roth, A., & Hauck, C. R. 2011, "Neisseria meningitidis has two independent modes of recognizing its human receptor CEACAM1", *PLoS.One.,* vol. 6, no. 1, pp. e14609

165. Ladhani, S. N., Lucidarme, J., Newbold, L. S., Gray, S. J., Carr, A. D., Findlow, J., Ramsay, M. E., Kaczmarski, E. B., & Borrow, R. 2012, "Invasive meningococcal capsular group Y disease, England and Wales, 2007-2009", *Emerg.Infect.Dis.,* vol. 18, no. 1, pp. 63-70

166. Laher, G., Balmer, P., Gray, S. J., Dawson, M., Kaczmarski, E. B., & Borrow, R. 2006, "Development and evaluation of a rapid multianalyte particle-based flow cytometric assay for the quantification of meningococcal serogroup B-specific IgM antibodies in sera for nonculture case confirmation", *FEMS Immunol.Med.Microbiol.,* vol. 48, no. 1, pp. 34-43

167. Lal, G., Balmer, P., Joseph, H., Dawson, M., & Borrow, R. 2004, "Development and evaluation of a tetraplex flow cytometric assay for quantitation of serum antibodies to Neisseria meningitidis serogroups A, C, Y, and W-135", *Clin.Diagn.Lab Immunol.,* vol. 11, no. 2, pp. 272-279

168. Lal, G., Balmer, P., Stanford, E., Martin, S., Warrington, R., & Borrow, R. 2005, "Development and validation of a nonaplex assay for the simultaneous quantitation of antibodies to nine Streptococcus pneumoniae serotypes", *J.Immunol.Methods,* vol. 296, no. 1-2, pp. 135-147

169. Lash, G. E., Scaife, P. J., Innes, B. A., Otun, H. A., Robson, S. C., Searle, R. F., & Bulmer, J. N. 2006, "Comparison of three multiplex cytokine analysis systems: Luminex, SearchLight and FAST Quant", *J.Immunol.Methods,* vol. 309, no. 1-2, pp. 205-208

170. Lauer, S. A. & Nolan, J. P. 2002, "Development and characterization of Ni-NTA-bearing microspheres", *Cytometry,* vol. 48, no. 3, pp. 136-145

171. Leach, A., Twumasi, P. A., Kumah, S., Banya, W. S., Jaffar, S., Forrest, B. D., Granoff, D. M., LiButti, D. E., Carlone, G. M., Pais, L. B., Broome, C. V., & Greenwood, B. M. 1997, "Induction of immunologic memory in Gambian children by vaccination in infancy with a group A plus group C meningococcal polysaccharide-protein conjugate vaccine", *J.Infect.Dis.,* vol. 175, no. 1, pp. 200-204

172. Lennon, D. 2008, "The role of MeNZB vaccination in controlling the New Zealand meningococcal epidemic", *N.Z.Med.J.,* vol. 121, no. 1270, pp. 110-112

173. Lewis, L. A., Sung, M. H., Gipson, M., Hartman, K., & Dyer, D. W. 1998, "Transport of intact porphyrin by HpuAB, the hemoglobin-haptoglobin utilization system of Neisseria meningitidis", *The Journal of Bacteriology,* vol. 180, no. 22, pp. 6043-6047

174. Lewis, L. A., Vu, D. M., Vasudhev, S., Shaughnessy, J., Granoff, D. M., & Ram, S. 2013, "Factor H-dependent alternative pathway inhibition mediated by porin B contributes to virulence of Neisseria meningitidis", *MBio.,* vol. 4, no. 5, pp. e00339-13

175. Leyva, A., Sanchez, J. C., Alvarez, D., Perez, B., Lopez, L., Paz, S., Torres, E., Gonzalez, T., Font, M., Hernandez, N., & Valdes, R. 2011, "Novel and sensitive ELISA for the rapid quantification of recombinant p64K protein", *J.Pharm.Biomed.Anal.,* vol. 55, no. 3, pp. 403-408

176. Lieberman, J. M., Chiu, S. S., Wong, V. K., Partidge, S., Chang, S. J., Chiu, C. Y., Gheesling, L. L., Carlone, G. M., & Ward, J. I. 1996, "Safety and immunogenicity of a serogroups A/C Neisseria meningitidis oligosaccharide-protein conjugate vaccine in young children. A randomized controlled trial", *JAMA,* vol. 275, no. 19, pp. 1499-1503

177. Liu, T. Y., Gotschlich, E. C., Dunne, F. T., & Jonssen, E. K. 1971, "Studies on the meningococcal polysaccharides. II. Composition and chemical properties of the group B and group C polysaccharide", *J.Biol.Chem.,* vol. 246, no. 15, pp. 4703-4712

178. Liu, Z. Q. & Yang, P. C. 2012, "Construction of pET-32 alpha (+) Vector for Protein Expression and Purification", *N.Am.J.Med.Sci.,* vol. 4, no. 12, pp. 651-655

179. Loughran, S. T. & Walls, D. 2011, "Purification of poly-histidine-tagged proteins", *Methods Mol.Biol.,* vol. 681, pp. 311-335

180. Lucidarme, J., Newbold, L. S., Findlow, J., Gilchrist, S., Gray, S. J., Carr, A. D., Hewitt, J., Kaczmarski, E. B., & Borrow, R. 2011, "Molecular targets in meningococci: efficient routine characterization and optimal outbreak investigation in conjunction with routine surveillance of the meningococcal group B vaccine candidate, fHBP", *Clin.Vaccine Immunol.,* vol. 18, no. 2, pp. 194-202

181. Luckey, M. 2008, "Membrane Structural Biology,",

182. Luijkx, T. A., van, D. H., Hamstra, H. J., Kuipers, B., van der Ley, P., van, A. L., & van den Dobbelsteen, G. 2003, "Relative immunogenicity of PorA subtypes in a multivalent Neisseria meningitidis vaccine is not dependent on presentation form", *Infect.Immun.,* vol. 71, no. 11, pp. 6367-6371

183. MacLennan, J., Kafatos, G., Neal, K., Andrews, N., Cameron, J. C., Roberts, R., Evans, M. R., Cann, K., Baxter, D. N., Maiden, M. C., & Stuart, J. M. 2006, "Social behavior and meningococcal carriage in British teenagers", *Emerg.Infect.Dis.,* vol. 12, no. 6, pp. 950-957

184. Maiden, M. C. 2006, "Multilocus sequence typing of bacteria", *Annu.Rev.Microbiol.,* vol. 60, pp. 561-588

185. Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M., & Spratt, B. G. 1998, "Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms", *Proc.Natl.Acad.Sci.U.S.A,* vol. 95, no. 6, pp. 3140-3145

186. Maiden, M. C., Ibarz-Pavon, A. B., Urwin, R., Gray, S. J., Andrews, N. J., Clarke, S. C., Walker, A. M., Evans, M. R., Kroll, J. S., Neal, K. R., Ala'Aldeen, D. A., Crook, D. W., Cann, K., Harrison, S., Cunningham, R., Baxter, D., Kaczmarski, E., MacLennan, J., Cameron, J. C., & Stuart, J. M. 2008, "Impact of meningococcal serogroup C conjugate vaccines on carriage and herd immunity", *J.Infect.Dis.,* vol. 197, no. 5, pp. 737-743

187. Maiden, M. C. & Stuart, J. M. 2002, "Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination", *Lancet,* vol. 359, no. 9320, pp. 1829-1831

188. Maiden, M. C., Suker, J., McKenna, A. J., Bygraves, J. A., & Feavers, I. M. 1991, "Comparison of the class 1 outer membrane proteins of eight serological reference strains of Neisseria meningitidis", *Mol.Microbiol.,* vol. 5, no. 3, pp. 727-736

189. Martin, D., McCallum, L., Glennie, A., Ruijne, N., Blatchford, P., O'Hallahan, J., & Oster, P. 2005, "Validation of the serum bactericidal assay for measurement of functional antibodies against group B meningococci associated with vaccine trials", *Vaccine,* vol. 23, no. 17-18, pp. 2218-2221

190. Martin, D. R., Ruijne, N., McCallum, L., O'Hallahan, J., & Oster, P. 2006, "The VR2 epitope on the PorA P1.7-2,4 protein is the major target for the immune response elicited by the strain-specific group B meningococcal vaccine MeNZB", *Clin.Vaccine Immunol.,* vol. 13, no. 4, pp. 486-491

191. Martin, D. R., Walker, S. J., Baker, M. G., & Lennon, D. R. 1998, "New Zealand epidemic of meningococcal disease identified by a strain with phenotype B:4:P1.4", *J.Infect.Dis.,* vol. 177, no. 2, pp. 497-500

192. Martin, S. L., Borrow, R., van der, L. P., Dawson, M., Fox, A. J., & Cartwright, K. A. 2000, "Effect of sequence variation in meningococcal PorA outer membrane protein on the effectiveness of a hexavalent PorA outer membrane vesicle vaccine", *Vaccine,* vol. 18, no. 23, pp. 2476-2481

193. Martins, T. B., Augustine, N. H., & Hill, H. R. 2006, "Development of a multiplexed fluorescent immunoassay for the quantitation of antibody responses to group A streptococci", *J.Immunol.Methods,* vol. 316, no. 1-2, pp. 97-106

194. Martins, T. B., Jaskowski, T. D., Tebo, A., & Hill, H. R. 2009, "Development of a multiplexed fluorescent immunoassay for the quantitation of antibody responses to four Neisseria meningitidis serogroups", *J.Immunol.Methods,* vol. 342, no. 1-2, pp. 98-105

195. Masignani, V., Comanducci, M., Giuliani, M. M., Bambini, S., du-Bobie, J., Arico, B., Brunelli, B., Pieri, A., Santini, L., Savino, S., Serruto, D., Litt, D., Kroll, S., Welsch, J. A., Granoff, D. M., Rappuoli, R., & Pizza, M. 2003, "Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870", *J.Exp.Med.,* vol. 197, no. 6, pp. 789-799

196. Maslanka, S. E., Gheesling, L. L., LiButti, D. E., Donaldson, K. B., Harakeh, H. S., Dykes, J. K., Arhin, F. F., Devi, S. J., Frasch, C. E., Huang, J. C., Kriz-Kuzemenska, P., Lemmon, R. D., Lorange, M., Peeters, C. C., Quataert, S., Tai, J. Y., & Carlone, G. M. 1997, "Standardization and a multilaboratory comparison of Neisseria meningitidis serogroup A and C serum bactericidal assays. The Multilaboratory Study Group", *Clin.Diagn.Lab Immunol.,* vol. 4, no. 2, pp. 156-167

197. Maslanka, S. E., Tappero, J. W., Plikaytis, B. D., Brumberg, R. S., Dykes, J. K., Gheesling, L. L., Donaldson, K. B., Schuchat, A., Pullman, J., Jones, M., Bushmaker, J., & Carlone, G. M. 1998, "Age-dependent Neisseria meningitidis serogroup C class-specific antibody concentrations and bactericidal titers in sera from young children from Montana immunized with a licensed polysaccharide vaccine", *Infect.Immun.,* vol. 66, no. 6, pp. 2453-2459

198. McGuinness, B., Barlow, A. K., Clarke, I. N., Farley, J. E., Anilionis, A., Poolman, J. T., & Heckels, J. E. 1990, "Deduced amino acid sequences of class 1 protein (PorA) from three strains of Neisseria meningitidis. Synthetic peptides define the epitopes responsible for serosubtype specificity", *J.Exp.Med.,* vol. 171, no. 6, pp. 1871-1882

199. McGuinness, B. T., Lambden, P. R., & Heckels, J. E. 1993, "Class 1 outer membrane protein of Neisseria meningitidis: epitope analysis of the antigenic diversity between strains, implications for subtype definition and molecular epidemiology", *Mol.Microbiol.,* vol. 7, no. 4, pp. 505-514

200. McNeil, L. K., Zagursky, R. J., Lin, S. L., Murphy, E., Zlotnick, G. W., Hoiseth, S. K., Jansen, K. U., & Anderson, A. S. 2013, "Role of factor H binding protein in Neisseria meningitidis virulence and its potential as a vaccine candidate to broadly protect against meningococcal disease", *Microbiol.Mol.Biol.Rev.,* vol. 77, no. 2, pp. 234-252

201. Medini, D., Serruto, D., Parkhill, J., Relman, D. A., Donati, C., Moxon, R., Falkow, S., & Rappuoli, R. 2008, "Microbiology in the post-genomic era", *Nat.Rev.Microbiol.,* vol. 6, no. 6, pp. 419-430

202. Mihalcu, F., Iacob, A., & Levenet, I. 1993, "Epidemiology of meningococcal meningitis in Romania 1971-1991", *Roum.Arch.Microbiol.Immunol.,* vol. 52, no. 1, pp. 57-65

203. Milagres, L. G., Gorla, M. C., Sacchi, C. T., & Rodrigues, M. M. 1998, "Specificity of bactericidal antibody response to serogroup B meningococcal strains in Brazilian

children after immunization with an outer membrane vaccine", *Infect.Immun.,* vol. 66, no. 10, pp. 4755-4761

204. Milagres, L. G., Ramos, S. R., Sacchi, C. T., Melles, C. E., Vieira, V. S., Sato, H., Brito, G. S., Moraes, J. C., & Frasch, C. E. 1994, "Immune response of Brazilian children to a Neisseria meningitidis serogroup B outer membrane protein vaccine: comparison with efficacy", *Infect.Immun.,* vol. 62, no. 10, pp. 4419-4424

205. Miller, E., Salisbury, D., & Ramsay, M. 2001, "Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story", *Vaccine,* vol. 20 Suppl 1, pp. S58-S67

206. Minetti, C. A., Tai, J. Y., Blake, M. S., Pullen, J. K., Liang, S. M., & Remeta, D. P. 1997, "Structural and functional characterization of a recombinant PorB class 2 protein from Neisseria meningitidis. Conformational stability and porin activity", *J.Biol.Chem.,* vol. 272, no. 16, pp. 10710-10720

207. Morales-Aza, B., Glennie, S. J., Garcez, T. P., Davenport, V., Johnston, S. L., Williams, N. A., & Heyderman, R. S. 2009, "Impaired maintenance of naturally acquired T-cell memory to the meningococcus in patients with B-cell immunodeficiency", *Blood,* vol. 113, no. 18, pp. 4206-4212

208. Morley, S. L. & Pollard, A. J. 2001, "Vaccine prevention of meningococcal disease, coming soon?", *Vaccine,* vol. 20, no. 5-6, pp. 666-687

209. Morton, D. J. & Williams, P. 1990, "Siderophore-independent acquisition of transferrin-bound iron by Haemophilus influenzae type b", *J.Gen.Microbiol.,* vol. 136, no. 5, pp. 927-933

210. Munkley, A., Tinsley, C. R., Virji, M., & Heckels, J. E. 1991, "Blocking of bactericidal killing of Neisseria meningitidis by antibodies directed against class 4 outer membrane protein", *Microb.Pathog.,* vol. 11, no. 6, pp. 447-452

211. Murphy, E., Andrew, L., Lee, K. L., Dilts, D. A., Nunez, L., Fink, P. S., Ambrose, K., Borrow, R., Findlow, J., Taha, M. K., Deghmane, A. E., Kriz, P., Musilek, M., Kalmusova, J., Caugant, D. A., Alvestad, T., Mayer, L. W., Sacchi, C. T., Wang, X., Martin, D., von, G. A., du, P. M., Klugman, K. P., Anderson, A. S., Jansen, K. U., Zlotnick, G. W., & Hoiseth, S. K. 2009, "Sequence diversity of the factor H binding protein vaccine candidate in epidemiologically relevant strains of serogroup B Neisseria meningitidis", *J.Infect.Dis.,* vol. 200, no. 3, pp. 379-389

212. Nolan, T., Lambert, S., Roberton, D., Marshall, H., Richmond, P., Streeton, C., Poolman, J., & Boutriau, D. 2007, "A novel combined Haemophilus influenzae type b-Neisseria meningitidis serogroups C and Y-tetanus-toxoid conjugate vaccine is immunogenic and induces immune memory when co-administered with DTPa-HBV-IPV and conjugate pneumococcal vaccines in infants", *Vaccine,* vol. 25, no. 51, pp. 8487-8499

213. Noronha, C. P., Struchiner, C. J., & Halloran, M. E. 1995, "Assessment of the direct effectiveness of BC meningococcal vaccine in Rio de Janeiro, Brazil: a case-control study", *Int.J.Epidemiol.,* vol. 24, no. 5, pp. 1050-1057

214. Oftung, F., Naess, L. M., Wetzler, L. M., Korsvold, G. E., Aase, A., Hoiby, E. A., Dalseg, R., Holst, J., Michaelsen, T. E., & Haneberg, B. 1999, "Antigen-specific T-cell responses in humans after intranasal immunization with a meningococcal

serogroup B outer membrane vesicle vaccine", *Infect.Immun.,* vol. 67, no. 2, pp. 921-927

215. Osborne, K., Gay, N., Hesketh, L., Morgan-Capner, P., & Miller, E. 2000, "Ten years of serological surveillance in England and Wales: methods, results, implications and action", *Int.J.Epidemiol.,* vol. 29, no. 2, pp. 362-368

216. Oster, P., Lennon, D., O'Hallahan, J., Mulholland, K., Reid, S., & Martin, D. 2005, "MeNZB: a safe and highly immunogenic tailor-made vaccine against the New Zealand Neisseria meningitidis serogroup B disease epidemic strain", *Vaccine,* vol. 23, no. 17-18, pp. 2191-2196

217. Oster, P., O'Hallahan, J., Aaberge, I., Tilman, S., Ypma, E., & Martin, D. 2007, "Immunogenicity and safety of a strain-specific MenB OMV vaccine delivered to under 5-year olds in New Zealand", *Vaccine,* vol. 25, no. 16, pp. 3075-3079

218. Pace, D. 2010, "Novel quadrivalent meningococcal A, C, W-135 and Y glycoconjugate vaccine for the broader protection of adolescents and adults", *Future.Microbiol.,* vol. 5, no. 11, pp. 1629-1640

219. Pace, D. & Pollard, A. J. 2012, "Meningococcal disease: clinical presentation and sequelae", *Vaccine,* vol. 30 Suppl 2, pp. B3-B9

220. Patel, M. S., Merianos, A., Hanna, J. N., Vartto, K., Tait, P., Morey, F., & Jayathissa, S. 1993, "Epidemic meningococcal meningitis in central Australia, 1987-1991", *Med.J.Aust.,* vol. 158, no. 5, pp. 336-340

221. Peeters, C. C., Claassen, I. J., Schuller, M., Kersten, G. F., van, d., V, & Poolman, J. T. 1999, "Immunogenicity of various presentation forms of PorA outer membrane protein of Neisseria meningitidis in mice", *Vaccine,* vol. 17, no. 20-21, pp. 2702-2712

222. Peeters, C. C., Rumke, H. C., Sundermann, L. C., Rouppe van der Voort EM, Meulenbelt, J., Schuller, M., Kuipers, A. J., van der, L. P., & Poolman, J. T. 1996, "Phase I clinical trial with a hexavalent PorA containing meningococcal outer membrane vesicle vaccine", *Vaccine,* vol. 14, no. 10, pp. 1009-1015

223. Peltola, H. 1983, "Meningococcal disease: still with us", *Rev.Infect.Dis.,* vol. 5, no. 1, pp. 71-91

224. Peltola, H., Jonsdottir, K., Lystad, A., Sievers, C. J., & Kallings, I. 1982, "Meningococcal disease in Scandinavia", *Br.Med.J.(Clin.Res.Ed),* vol. 284, no. 6329, pp. 1618-1621

225. Peltola, H., Kataja, J. M., & Makela, P. H. 1982, "Shift in the age-distribution of meningococcal disease as predictor of an epidemic?", *Lancet,* vol. 2, no. 8298, pp. 595-597

226. Peltola, H., Kilpi, T., & Anttila, M. 1992, "Rapid disappearance of Haemophilus influenzae type b meningitis after routine childhood immunisation with conjugate vaccines", *Lancet,* vol. 340, no. 8819, pp. 592-594

227. Pichichero, M., Casey, J., Blatter, M., Rothstein, E., Ryall, R., Bybel, M., Gilmet, G., & Papa, T. 2005, "Comparative trial of the safety and immunogenicity of quadrivalent (A, C, Y, W-135) meningococcal polysaccharide-diphtheria conjugate vaccine versus quadrivalent polysaccharide vaccine in two- to ten-year-old children", *Pediatr.Infect.Dis.J.,* vol. 24, no. 1, pp. 57-62

228. Pichichero, M. E. 2009, "Booster vaccinations: can immunologic memory outpace disease pathogenesis?", *Pediatrics,* vol. 124, no. 6, pp. 1633-1641

229. Pickering, J. W., Martins, T. B., Greer, R. W., Schroder, M. C., Astill, M. E., Litwin, C. M., Hildreth, S. W., & Hill, H. R. 2002, "A multiplexed fluorescent microsphere immunoassay for antibodies to pneumococcal capsular polysaccharides", *Am.J.Clin.Pathol.,* vol. 117, no. 4, pp. 589-596

230. Pickering, J. W., Martins, T. B., Schroder, M. C., & Hill, H. R. 2002, "Comparison of a multiplex flow cytometric assay with enzyme-linked immunosorbent assay for quantitation of antibodies to tetanus, diphtheria, and Haemophilus influenzae Type b", *Clin.Diagn.Lab Immunol.,* vol. 9, pp. 872-876

231. Pillai, S., Howell, A., Alexander, K., Bentley, B. E., Jiang, H. Q., Ambrose, K., Zhu, D., & Zlotnick, G. 2005, "Outer membrane protein (OMP) based vaccine for Neisseria meningitidis serogroup B", *Vaccine,* vol. 23, no. 17-18, pp. 2206-2209

232. Pinto, V. B., Moran, E. E., Cruz, F., Wang, X. M., Fridman, A., Zollinger, W. D., Przysiecki, C. T., & Burden, R. 2011, "An experimental outer membrane vesicle vaccine from N. meningitidis serogroup B strains that induces serum bactericidal activity to multiple serogroups", *Vaccine,* vol. 29, no. 44, pp. 7752-7758

233. Pizza, M., Scarlato, V., Masignani, V., Giuliani, M. M., Arico, B., Comanducci, M., Jennings, G. T., Baldi, L., Bartolini, E., Capecchi, B., Galeotti, C. L., Luzzi, E., Manetti, R., Marchetti, E., Mora, M., Nuti, S., Ratti, G., Santini, L., Savino, S., Scarselli, M., Storni, E., Zuo, P., Broeker, M., Hundt, E., Knapp, B., Blair, E., Mason, T., Tettelin, H., Hood, D. W., Jeffries, A. C., Saunders, N. J., Granoff, D. M., Venter, J. C., Moxon, E. R., Grandi, G., & Rappuoli, R. 2000, "Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing", *Science,* vol. 287, no. 5459, pp. 1816-1820

234. Platt, A., MacLeod, H., Massari, P., Liu, X., & Wetzler, L. 2013, "In vivo and in vitro characterization of the immune stimulating activity of the Neisserial porin PorB", *PLoS.One.,* vol. 8, no. 12, pp. e82171

235. Plikaytis, B. D., Holder, P. F., Pais, L. B., Maslanka, S. E., Gheesling, L. L., & Carlone, G. M. 1994, "Determination of parallelism and nonparallelism in bioassay dilution curves", *J.Clin.Microbiol.,* vol. 32, pp. 2441-2447

236. Plikaytis, B. D., Stella, M., Boccadifuoco, G., DeTora, L. M., Agnusdei, M., Santini, L., Brunelli, B., Orlandi, L., Simmini, I., Giuliani, M., Ledroit, M., Hong, E., Taha, M. K., Ellie, K., Rajam, G., Carlone, G. M., Claus, H., Vogel, U., Borrow, R., Findlow, J., Gilchrist, S., Stefanelli, P., Fazio, C., Carannante, A., Oksnes, J., Fritzsonn, E., Klem, A. M., Caugant, D. A., Abad, R., Vazquez, J. A., Rappuoli, R., Pizza, M., Donnelly, J. J., & Medini, D. 2012, "Interlaboratory standardization of the sandwich enzyme-linked immunosorbent assay designed for MATS, a rapid, reproducible method for estimating the strain coverage of investigational vaccines", *Clin.Vaccine Immunol.,* vol. 19, no. 10, pp. 1609-1617

237. Pollard, A. J. & Frasch, C. 2001, "Development of natural immunity to Neisseria meningitidis", *Vaccine,* vol. 19, no. 11-12, pp. 1327-1346

238. Pollard, A. J., Galassini, R., van, d., V, Booy, R., Langford, P., Nadel, S., Ison, C., Kroll, J. S., Poolman, J., & Levin, M. 1999, "Humoral immune responses to Neisseria meningitidis in children", *Infect.Immun.,* vol. 67, no. 5, pp. 2441-2451

239. Poolman, J. T., Kriz-Kuzemenska, P., Ashton, F., Bibb, W., Dankert, J., Demina, A., Froholm, L. O., Hassan-King, M., Jones, D. M., Lind, I., & . 1995, "Serotypes and subtypes of Neisseria meningitidis: results of an international study comparing sensitivities and specificities of monoclonal antibodies", *Clin.Diagn.Lab Immunol.*, vol. 2, no. 1, pp. 69-72

240. Poolman, J. T., Lind, I., Jonsdottir, K., Froholm, L. O., Jones, D. M., & Zanen, H. C. 1986, "Meningococcal serotypes and serogroup B disease in north-west Europe", *Lancet*, vol. 2, no. 8506, pp. 555-558

241. Popovic, T., Schmink, S., Rosenstein, N. A., Ajello, G. W., Reeves, M. W., Plikaytis, B., Hunter, S. B., Ribot, E. M., Boxrud, D., Tondella, M. L., Kim, C., Noble, C., Mothershed, E., Besser, J., & Perkins, B. A. 2001, "Evaluation of pulsed-field gel electrophoresis in epidemiological investigations of meningococcal disease outbreaks caused by Neisseria meningitidis serogroup C", *J.Clin.Microbiol.*, vol. 39, no. 1, pp. 75-85

242. Prince, H. E., Lape-Nixon, M., & Matud, J. 2006, "Evaluation of a tetraplex microsphere assay for Bordetella pertussis antibodies", *Clin.Vaccine Immunol.*, vol. 13, pp. 266-270

243. Prinz, T. & Tommassen, J. 2000, "Association of iron-regulated outer membrane proteins of Neisseria meningitidis with the RmpM (class 4) protein", *FEMS Microbiol.Lett.*, vol. 183, no. 1, pp. 49-53

244. Racloz, V. N. & Luiz, S. J. 2010, "The elusive meningococcal meningitis serogroup: a systematic review of serogroup B epidemiology", *BMC.Infect.Dis.*, vol. 10, pp. 175

245. Raghunathan, P. L., Bernhardt, S. A., & Rosenstein, N. E. 2004, "Opportunities for control of meningococcal disease in the United States", *Annu.Rev.Med.*, vol. 55, pp. 333-353

246. Ramsay, M. E., Andrews, N. J., Trotter, C. L., Kaczmarski, E. B., & Miller, E. 2003, "Herd immunity from meningococcal serogroup C conjugate vaccination in England: database analysis", *BMJ*, vol. 326, no. 7385, pp. 365-366

247. Rappuoli, R. 2000, "Reverse vaccinology", *Curr.Opin.Microbiol.*, vol. 3, no. 5, pp. 445-450

248. Reisinger, K. S., Baxter, R., Block, S. L., Shah, J., Bedell, L., & Dull, P. M. 2009, "Quadrivalent meningococcal vaccination of adults: phase III comparison of an investigational conjugate vaccine, MenACWY-CRM, with the licensed vaccine, Menactra", *Clin.Vaccine Immunol.*, vol. 16, no. 12, pp. 1810-1815

249. Reller, L. B., MacGregor, R. R., & Beaty, H. N. 1973, "Bactericidal antibody after colonization with Neisseria meningitidis", *J.Infect.Dis.*, vol. 127, no. 1, pp. 56-62

250. Richmond, P., Borrow, R., Goldblatt, D., Findlow, J., Martin, S., Morris, R., Cartwright, K., & Miller, E. 2001, "Ability of 3 different meningococcal C conjugate vaccines to induce immunologic memory after a single dose in UK toddlers", *J.Infect.Dis.*, vol. 183, no. 1, pp. 160-163

251. Richmond, P., Borrow, R., Miller, E., Clark, S., Sadler, F., Fox, A., Begg, N., Morris, R., & Cartwright, K. 1999, "Meningococcal serogroup C conjugate vaccine is immunogenic in infancy and primes for memory", *J.Infect.Dis.*, vol. 179, no. 6, pp. 1569-1572

252. Roberts, R. B. 1970, "The relationship between group A and group C meningococcal polysaccharides and serum opsonins in man", *J.Exp.Med.,* vol. 131, no. 3, pp. 499-513

253. Rosenqvist, E., Hoiby, E. A., Wedege, E., Bryn, K., Kolberg, J., Klem, A., Ronnild, E., Bjune, G., & Nokleby, H. 1995, "Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine", *Infect.Immun.,* vol. 63, no. 12, pp. 4642-4652

254. Rosenqvist, E., Tjade, T., Froholm, L. O., & Frasch, C. E. 1983, "An ELISA study of the antibody response after vaccination with a combined meningococcal group B polysaccharide and serotype 2 outer membrane protein vaccine", *NIPH Ann.,* vol. 6, no. 2, pp. 139-149

255. Rosenstein, N. E., Fischer, M., & Tappero, J. W. 2001, "Meningococcal vaccines", *Infect.Dis.Clin.North Am.,* vol. 15, no. 1, pp. 155-169

256. Rosenstein, N. E., Perkins, B. A., Stephens, D. S., Lefkowitz, L., Cartter, M. L., Danila, R., Cieslak, P., Shutt, K. A., Popovic, T., Schuchat, A., Harrison, L. H., & Reingold, A. L. 1999, "The changing epidemiology of meningococcal disease in the United States, 1992-1996", *J.Infect.Dis.,* vol. 180, no. 6, pp. 1894-1901

257. Rosenstein, N. E., Perkins, B. A., Stephens, D. S., Popovic, T., & Hughes, J. M. 2001, "Meningococcal disease", *N.Engl.J.Med.,* vol. 344, no. 18, pp. 1378-1388

258. Russell, J. E., Jolley, K. A., Feavers, I. M., Maiden, M. C., & Suker, J. 2004, "PorA variable regions of Neisseria meningitidis", *Emerg.Infect.Dis.,* vol. 10, no. 4, pp. 674-678

259. Russell, J. E., Urwin, R., Gray, S. J., Fox, A. J., Feavers, I. M., & Maiden, M. C. 2008, "Molecular epidemiology of meningococcal disease in England and Wales 1975-1995, before the introduction of serogroup C conjugate vaccines", *Microbiology,* vol. 154, no. Pt 4, pp. 1170-1177

260. Sacchi, C. T., Lemos, A. P., Brandt, M. E., Whitney, A. M., Melles, C. E., Solari, C. A., Frasch, C. E., & Mayer, L. W. 1998, "Proposed standardization of Neisseria meningitidis PorA variable-region typing nomenclature", *Clin.Diagn.Lab Immunol.,* vol. 5, no. 6, pp. 845-855

261. Sacchi, C. T., Lemos, A. P., Whitney, A. M., Solari, C. A., Brandt, M. E., Melles, C. E., Frasch, C. E., & Mayer, L. W. 1998, "Correlation between serological and sequencing analyses of the PorB outer membrane protein in the Neisseria meningitidis serotyping system", *Clin.Diagn.Lab Immunol.,* vol. 5, no. 3, pp. 348-354

262. Sacchi, C. T., Pessoa, L. L., Ramos, S. R., Milagres, L. G., Camargo, M. C., Hidalgo, N. T., Melles, C. E., Caugant, D. A., & Frasch, C. E. 1992, "Ongoing group B Neisseria meningitidis epidemic in Sao Paulo, Brazil, due to increased prevalence of a single clone of the ET-5 complex", *J.Clin.Microbiol.,* vol. 30, no. 7, pp. 1734-1738

263. Sadarangani, M. & Pollard, A. J. 2010, "Serogroup B meningococcal vaccines-an unfinished story", *Lancet Infect.Dis.,* vol. 10, no. 2, pp. 112-124

264. Saleem, M., Moore, J., & Derrick, J. P. 2012, "Expression, purification, and crystallization of neisserial outer membrane proteins", *Methods Mol.Biol.,* vol. 799, pp. 91-106

265. Saleem, M., Prince, S. M., Patel, H., Chan, H., Feavers, I. M., & Derrick, J. P. 2012, "Refolding, purification and crystallization of the FrpB outer membrane iron

transporter from Neisseria meningitidis", *Acta Crystallogr.Sect.F.Struct.Biol.Cryst.Commun.,* vol. 68, no. Pt 2, pp. 231-235

266. Saleem, M., Prince, S. M., Rigby, S. E., Imran, M., Patel, H., Chan, H., Sanders, H., Maiden, M. C., Feavers, I. M., & Derrick, J. P. 2013, "Use of a molecular decoy to segregate transport from antigenicity in the FrpB iron transporter from Neisseria meningitidis", *PLoS.One.,* vol. 8, no. 2, pp. e56746

267. Sandbu, S., Feiring, B., Oster, P., Helland, O. S., Bakke, H. S., Naess, L. M., Aase, A., Aaberge, I. S., Kristoffersen, A. C., Rydland, K. M., Tilman, S., Nokleby, H., & Rosenqvist, E. 2007, "Immunogenicity and safety of a combination of two serogroup B meningococcal outer membrane vesicle vaccines", *Clin.Vaccine Immunol.,* vol. 14, no. 9, pp. 1062-1069

268. Sarkari, J., Pandit, N., Moxon, E. R., & Achtman, M. 1994, "Variable expression of the Opc outer membrane protein in Neisseria meningitidis is caused by size variation of a promoter containing poly-cytidine", *Mol.Microbiol.,* vol. 13, no. 2, pp. 207-217

269. Saukkonen, K., Abdillahi, H., Poolman, J. T., & Leinonen, M. 1987, "Protective efficacy of monoclonal antibodies to class 1 and class 3 outer membrane proteins of Neisseria meningitidis B:15:P1.16 in infant rat infection model: new prospects for vaccine development", *Microb.Pathog.,* vol. 3, no. 4, pp. 261-267

270. Schlech, W. F. I., Ward, J. I., Band, J. D., Hightower, A., Fraser, D. W., & Broome, C. V. 1985, "Bacterial meningitis in the United States, 1978 through 1981. The National Bacterial Meningitis Surveillance Study", *JAMA,* vol. 253, no. 12, pp. 1749-1754

271. Schmitt, J., Hess, H., & Stunnenberg, H. G. 1993, "Affinity purification of histidine-tagged proteins", *Mol.Biol.Rep.,* vol. 18, no. 3, pp. 223-230

272. Schneider, M. C., Exley, R. M., Chan, H., Feavers, I., Kang, Y. H., Sim, R. B., & Tang, C. M. 2006, "Functional significance of factor H binding to Neisseria meningitidis", *J.Immunol.,* vol. 176, no. 12, pp. 7566-7575

273. Segal, S. & Pollard, A. J. 2004, "Vaccines against bacterial meningitis", *Br.Med.Bull.,* vol. 72, pp. 65-81

274. Sexton, K., Lennon, D., Oster, P., Crengle, S., Martin, D., Mulholland, K., Percival, T., Reid, S., Stewart, J., & O'Hallahan, J. 2004, "The New Zealand Meningococcal Vaccine Strategy: a tailor-made vaccine to combat a devastating epidemic", *N.Z.Med.J.,* vol. 117, no. 1200, pp. U1015

275. Shoma, S., Verkaik, N. J., de Vogel, C. P., Hermans, P. W., van, S. S., Mitchell, T. J., van, R. M., Hossain, S., Rahman, M., Endtz, H. P., van Wamel, W. J., & van, B. A. 2010, "Development of a multiplexed bead-based immunoassay for the simultaneous detection of antibodies to 17 pneumococcal proteins", *Eur.J.Clin.Microbiol.Infect.Dis.,*

276. Sierra, G. V., Campa, H. C., Varcacel, N. M., Garcia, I. L., Izquierdo, P. L., Sotolongo, P. F., Casanueva, G. V., Rico, C. O., Rodriguez, C. R., & Terry, M. H. 1991, "Vaccine against group B Neisseria meningitidis: protection trial and mass vaccination results in Cuba", *NIPH Ann.,* vol. 14, no. 2, pp. 195-207

277. Sikkema, D. J., Friedman, K. E., Corsaro, B., Kimura, A., Hildreth, S. W., Madore, D. V., & Quataert, S. A. 2000, "Relationship between serum bactericidal activity and serogroup-specific immunoglobulin G concentration for adults, toddlers, and

infants immunized with Neisseria meningitidis serogroup C vaccines", *Clin.Diagn.Lab Immunol.,* vol. 7, no. 5, pp. 764-768

278. Smits, G. P., van Gageldonk, P. G., Schouls, L. M., van der Klis, F. R., & Berbers, G. A. 2012, "Development of a bead-based multiplex immunoassay for simultaneous quantitative detection of IgG serum antibodies against measles, mumps, rubella, and varicella-zoster virus", *Clin.Vaccine Immunol.,* vol. 19, no. 3, pp. 396-400

279. Snape, M. D., Dawson, T., Oster, P., Evans, A., John, T. M., Ohene-Kena, B., Findlow, J., Yu, L. M., Borrow, R., Ypma, E., Toneatto, D., & Pollard, A. J. 2010, "Immunogenicity of two investigational serogroup B meningococcal vaccines in the first year of life: a randomized comparative trial", *Pediatr.Infect.Dis.J.,* vol. 29, no. 11, pp. e71-e79

280. Sotolongo Padron, F., Huergo, C. C., Gil, V. C., Diaz, E. M. F., Valdespino, I. E. C., & Gotera, N. G. 2007, "Cuban Meningococcal BC Vaccine: Experiences & Contributions from 20 Years of Application", *MEDICC.Rev.,* vol. 9, no. 1, pp. 16-22

281. Sparks, K. & Ballow, M. 1983, "The indirect ELISA for quantitation of specific antibody: analysis of antibody dilution curves", *Diagn.Immunol.,* vol. 1, no. 4, pp. 269-275

282. Stanton, M. C., Taylor-Robinson, D., Harris, D., Paize, F., Makwana, N., Hackett, S. J., Baines, P. B., Riordan, F. A., Marzouk, O., Thomson, A. P., Diggle, P. J., Hart, C. A., & Carrol, E. D. 2011, "Meningococcal disease in children in Merseyside, England: a 31 year descriptive study", *PLoS.One.,* vol. 6, no. 10, pp. e25957

283. Steeghs, L., den Hartog, R., den Boer, A., Zomer, B., Roholl, P., & van der Ley, P. 1998, "Meningitis bacterium is viable without endotoxin", *Nature,* vol. 392, no. 6675, pp. 449-450

284. Stein, D. M., Robbins, J., Miller, M. A., Lin, F. Y., & Schneerson, R. 2005, "Are antibodies to the capsular polysaccharide of *Neisseria meningitidis* group B and *Escherichia coli* K1 associated with immunopathology?", *Vaccine,* vol. 24(3), pp. 221-228

285. Stenger, R. M., Smits, M., Kuipers, B., Kessen, S. F., Boog, C. J., & van Els, C. A. 2011, "Fast, antigen-saving multiplex immunoassay to determine levels and avidity of mouse serum antibodies to pertussis, diphtheria, and tetanus antigens", *Clin.Vaccine Immunol.,* vol. 18, no. 4, pp. 595-603

286. Stephens, D. S. 1999, "Uncloaking the meningococcus: dynamics of carriage and disease", *Lancet,* vol. 353, no. 9157, pp. 941-942

287. Stuart, J. M., Cartwright, K. A., Dawson, J. A., Rickard, J., & Noah, N. D. 1988, "Risk factors for meningococcal disease: a case control study in south west England", *Community Med.,* vol. 10, no. 2, pp. 139-146

288. Stuart, J. M., Cartwright, K. A., Robinson, P. M., & Noah, N. D. 1989, "Effect of smoking on meningococcal carriage", *Lancet,* vol. 2, no. 8665, pp. 723-725

289. Su, E. L. & Snape, M. D. 2011, "A combination recombinant protein and outer membrane vesicle vaccine against serogroup B meningococcal disease", *Expert.Rev.Vaccines.,* vol. 10, no. 5, pp. 575-588

290. Suker, J., Feavers, I. M., Achtman, M., Morelli, G., Wang, J. F., & Maiden, M. C. 1994, "The porA gene in serogroup A meningococci: evolutionary stability and mechanism of genetic variation", *Mol.Microbiol.,* vol. 12, no. 2, pp. 253-265

291. Swartley, J. S., Liu, L. J., Miller, Y. K., Martin, L. E., Edupuganti, S., & Stephens, D. S. 1998, "Characterization of the gene cassette required for biosynthesis of the (alpha1-->6)-linked N-acetyl-D-mannosamine-1-phosphate capsule of serogroup A Neisseria meningitidis", *The Journal of Bacteriology,* vol. 180, no. 6, pp. 1533-1539

292. Swartley, J. S., Marfin, A. A., Edupuganti, S., Liu, L. J., Cieslak, P., Perkins, B., Wenger, J. D., & Stephens, D. S. 1997, "Capsule switching of Neisseria meningitidis", *Proc.Natl.Acad.Sci.U.S.A,* vol. 94, no. 1, pp. 271-276

293. Swartz, M. N. 2004, "Bacterial meningitis--a view of the past 90 years", *N.Engl.J.Med.,* vol. 351, no. 18, pp. 1826-1828

294. Taha, M. K. & Deghmane, A. E. 2012, "Vaccines targeting serogroup B meningococci", *Lancet Infect.Dis.,* vol. 12, no. 8, pp. 578-579

295. Tappero, J. W., Lagos, R., Ballesteros, A. M., Plikaytis, B., Williams, D., Dykes, J., Gheesling, L. L., Carlone, G. M., Hoiby, E. A., Holst, J., Nokleby, H., Rosenqvist, E., Sierra, G., Campa, C., Sotolongo, F., Vega, J., Garcia, J., Herrera, P., Poolman, J. T., & Perkins, B. A. 1999, "Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile", *JAMA,* vol. 281, no. 16, pp. 1520-1527

296. Thompson, E. A., Feavers, I. M., & Maiden, M. C. 2003, "Antigenic diversity of meningococcal enterobactin receptor FetA, a vaccine component", *Microbiology,* vol. 149, no. Pt 7, pp. 1849-1858

297. Tondella, M. L., Popovic, T., Rosenstein, N. E., Lake, D. B., Carlone, G. M., Mayer, L. W., & Perkins, B. A. 2000, "Distribution of Neisseria meningitidis serogroup B serosubtypes and serotypes circulating in the United States. The Active Bacterial Core Surveillance Team", *J.Clin.Microbiol.,* vol. 38, no. 9, pp. 3323-3328

298. Trotter, C., Findlow, J., Balmer, P., Holland, A., Barchha, R., Hamer, N., Andrews, N., Miller, E., & Borrow, R. 2007, "Seroprevalence of bactericidal and anti-outer membrane vesicle antibodies to Neisseria meningitidis group B in England", *Clin.Vaccine Immunol.,* vol. 14, no. 7, pp. 863-868

299. Trotter, C. L., Andrews, N. J., Kaczmarski, E. B., Miller, E., & Ramsay, M. E. 2004, "Effectiveness of meningococcal serogroup C conjugate vaccine 4 years after introduction", *Lancet,* vol. 364, no. 9431, pp. 365-367

300. Trotter, C. L., Borrow, R., Findlow, J., Holland, A., Frankland, S., Andrews, N. J., & Miller, E. 2008, "Seroprevalence of antibodies against serogroup C meningococci in England in the postvaccination era", *Clin.Vaccine Immunol.,* vol. 15, no. 11, pp. 1694-1698

301. Trotter, C. L., Edmunds, W. J., Ramsay, M. E., & Miller, E. 2006, "Modeling future changes to the meningococcal serogroup C conjugate (MCC) vaccine program in England and Wales", *Hum.Vaccin.,* vol. 2, no. 2, pp. 68-73

302. Trotter, C. L., Gay, N. J., & Edmunds, W. J. 2005, "Dynamic models of meningococcal carriage, disease, and the impact of serogroup C conjugate vaccination", *Am.J.Epidemiol.,* vol. 162, no. 1, pp. 89-100

303. Trotter, C. L., Gay, N. J., & Edmunds, W. J. 2006, "The natural history of meningococcal carriage and disease", *Epidemiol.Infect.,* vol. 134, no. 3, pp. 556-566

304. Trotter, C. L. & Ramsay, M. E. 2007, "Vaccination against meningococcal disease in Europe: review and recommendations for the use of conjugate vaccines", *FEMS Microbiol.Rev.,* vol. 31, no. 1, pp. 101-107

305. Tsai, C. M., Frasch, C. E., & Mocca, L. F. 1981, "Five structural classes of major outer membrane proteins in Neisseria meningitidis", *The Journal of Bacteriology,* vol. 146, no. 1, pp. 69-78

306. Tsai, C. M., Mocca, L. F., & Frasch, C. E. 1987, "Immunotype epitopes of Neisseria meningitidis lipooligosaccharide types 1 through 8", *Infect.Immun.,* vol. 55, no. 7, pp. 1652-1656

307. Tsai, J., Dyer, D. W., & Sparling, P. F. 1988, "Loss of transferrin receptor activity in Neisseria meningitidis correlates with inability to use transferrin as an iron source", *Infect.Immun.,* vol. 56, no. 12, pp. 3132-3138

308. Tzeng, Y. L., Datta, A. K., Strole, C. A., Lobritz, M. A., Carlson, R. W., & Stephens, D. S. 2005, "Translocation and surface expression of lipidated serogroup B capsular Polysaccharide in Neisseria meningitidis", *Infect.Immun.,* vol. 73, no. 3, pp. 1491-1505

309. Uli, L., Castellanos-Serra, L., Betancourt, L., Dominguez, F., Barbera, R., Sotolongo, F., Guillen, G., & Pajon, F. R. 2006, "Outer membrane vesicles of the VA-MENGOC-BC vaccine against serogroup B of Neisseria meningitidis: Analysis of protein components by two-dimensional gel electrophoresis and mass spectrometry", *Proteomics,* vol. 6, no. 11, pp. 3389-3399

310. Unkmeir, A., Kammerer, U., Stade, A., Hubner, C., Haller, S., Kolb-Maurer, A., Frosch, M., & Dietrich, G. 2002, "Lipooligosaccharide and polysaccharide capsule: virulence factors of Neisseria meningitidis that determine meningococcal interaction with human dendritic cells", *Infect.Immun.,* vol. 70, no. 5, pp. 2454-2462

311. Urwin, R., Feavers, I. M., Jones, D. M., Maiden, M. C., & Fox, A. J. 1998, "Molecular variation of meningococcal serotype 4 antigen genes", *Epidemiol.Infect.,* vol. 121, no. 1, pp. 95-101

312. Urwin, R., Fox, A. J., Musilek, M., Kriz, P., & Maiden, M. C. 1998, "Heterogeneity of the PorB protein in serotype 22 Neisseria meningitidis", *J.Clin.Microbiol.,* vol. 36, no. 12, pp. 3680-3682

313. Urwin, R., Holmes, E. C., Fox, A. J., Derrick, J. P., & Maiden, M. C. 2002, "Phylogenetic evidence for frequent positive selection and recombination in the meningococcal surface antigen PorB", *Mol.Biol.Evol.,* vol. 19, no. 10, pp. 1686-1694

314. Urwin, R., Russell, J. E., Thompson, E. A., Holmes, E. C., Feavers, I. M., & Maiden, M. C. 2004, "Distribution of surface protein variants among hyperinvasive meningococci: implications for vaccine design", *Infect.Immun.,* vol. 72, no. 10, pp. 5955-5962

315. van de Waterbeemd, B., Streefland, M., van der Ley, P., Zomer, B., van Dijken, H., Martens, D., Wijffels, R., & van der Pol, L. 2010, "Improved OMV vaccine against Neisseria meningitidis using genetically engineered strains and a detergent-free purification process", *Vaccine,* vol. 28, no. 30, pp. 4810-4816

316. van den Dobbelsteen, G. P., van Dijken, H. H., Pillai, S., & van Alphen, L. 2007, "Immunogenicity of a combination vaccine containing pneumococcal conjugates and meningococcal PorA OMVs", *Vaccine,* vol. 25, no. 13, pp. 2491-2496

317. van der Ende, A., Hopman, C. T., & Dankert, J. 2000, "Multiple mechanisms of phase variation of PorA in Neisseria meningitidis", *Infect.Immun.,* vol. 68, no. 12, pp. 6685-6690

318. van der Ley, P., Heckels, J. E., Virji, M., Hoogerhout, P., & Poolman, J. T. 1991, "Topology of outer membrane porins in pathogenic Neisseria spp", *Infect.Immun.,* vol. 59, no. 9, pp. 2963-2971

319. van der Ley, P. & Poolman, J. T. 1992, "Construction of a multivalent meningococcal vaccine strain based on the class 1 outer membrane protein", *Infect.Immun.,* vol. 60, no. 8, pp. 3156-3161

320. van der Ley, P., Steeghs, L., Hamstra, H. J., ten Hove, J., Zomer, B., & van Alphen L. 2001, "Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity", *Infect.Immun.,* vol. 69, no. 10, pp. 5981-5990

321. van der Ley, P. & van den Dobbelsteen, G. 2011, "Next-generation outer membrane vesicle vaccines against Neisseria meningitidis based on nontoxic LPS mutants", *Hum.Vaccin.,* vol. 7, no. 8, pp. 886-890

322. van der Ley, P., van der Biezen J., Sutmuller, R., Hoogerhout, P., & Poolman, J. T. 1996, "Sequence variability of FrpB, a major iron-regulated outer-membrane protein in the pathogenic neisseriae", *Microbiology,* vol. 142 (Pt 11), pp. 3269-3274

323. van der Ley, P., van der Biezen, J., & Poolman, J. T. 1995, "Construction of Neisseria meningitidis strains carrying multiple chromosomal copies of the porA gene for use in the production of a multivalent outer membrane vesicle vaccine", *Vaccine,* vol. 13, no. 4, pp. 401-407

324. van der Voort, E., van der Ley, P., van der Biezen, J., George, S., Tunnela, O., van Dijken, H., Kuipers, B., & Poolman, J. 1996, "Specificity of human bactericidal antibodies against PorA P1.7,16 induced with a hexavalent meningococcal outer membrane vesicle vaccine", *Infect.Immun.,* vol. 64, no. 7, pp. 2745-2751

325. van Gageldonk, P. G., van Schaijk, F. G., van der Klis, F. R., & Berbers, G. A. 2008, "Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus", *J.Immunol.Methods,* vol. 335, no. 1-2, pp. 79-89

326. van Gageldonk, P. G., von, H. C., van der Klis, F. R., & Berbers, G. A. 2011, "Improved specificity of a multiplex immunoassay for quantitation of anti-diphtheria toxin antibodies with the use of diphtheria toxoid", *Clin.Vaccine Immunol.,* vol. 18, no. 7, pp. 1183-1186

327. Vassiliadis, P., Kanellakis, A., & Papadakis, J. 1969, "Sulphadiazine-resistant group A meningococci isolated during the 1968 meningitis epidemic in Greece", *J.Hyg.(Lond),* vol. 67, no. 2, pp. 279-288

328. Vedros, N. A. 1987, "Development of meningococcal serogroups.", vol. 2, no. 4, pp. 33-37

329. Verkaik, N., Brouwer, E., Hooijkaas, H., van, B. A., & van, W. W. 2008, "Comparison of carboxylated and Penta-His microspheres for semi-quantitative measurement of antibody responses to His-tagged proteins", *J.Immunol.Methods,* vol. 335, no. 1-2, pp. 121-125

330. Vermont, C. L., van Dijken, H. H., Kuipers, A. J., van Limpt, C. J., Keijzers, W. C., van der, E. A., de, G. R., van, A. L., & van den Dobbelsteen, G. P. 2003, "Cross-reactivity of antibodies against PorA after vaccination with a meningococcal B outer membrane vesicle vaccine", *Infect.Immun.,* vol. 71, no. 4, pp. 1650-1655

331. Vesikari, T., Esposito, S., Prymula, R., Ypma, E., Kohl, I., Toneatto, D., Dull, P., & Kimura, A. 2013, "Immunogenicity and safety of an investigational multicomponent, recombinant, meningococcal serogroup B vaccine (4CMenB) administered concomitantly with routine infant and child vaccinations: results of two randomised trials", *Lancet,* vol. 381, no. 9869, pp. 825-835

332. Vignali, D. A. 2000, "Multiplexed particle-based flow cytometric assays", *J.Immunol.Methods,* vol. 243, no. 1-2, pp. 243-255

333. Virji, M., Makepeace, K., Ferguson, D. J., Achtman, M., & Moxon, E. R. 1993, "Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells", *Mol.Microbiol.,* vol. 10, no. 3, pp. 499-510

334. Virji, M., Makepeace, K., Ferguson, D. J., Achtman, M., Sarkari, J., & Moxon, E. R. 1992, "Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by Neisseria meningitidis", *Mol.Microbiol.,* vol. 6, no. 19, pp. 2785-2795

335. Virji, M., Makepeace, K., Ferguson, D. J., & Watt, S. M. 1996, "Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae", *Mol.Microbiol.,* vol. 22, no. 5, pp. 941-950

336. Virji, M., Watt, S. M., Barker, S., Makepeace, K., & Doyonnas, R. 1996, "The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of Neisseria meningitidis and Neisseria gonorrhoeae", *Mol.Microbiol.,* vol. 22, no. 5, pp. 929-939

337. Vogel, U., Claus, H., von Muller, L., Bunjes, D., Elias, J., & Frosch, M. 2004, "Bacteremia in an immunocompromised patient caused by a commensal Neisseria meningitidis strain harboring the capsule null locus (cnl)", *J.Clin.Microbiol.,* vol. 42, no. 7, pp. 2898-2901

338. Wang, J. F., Caugant, D. A., Li, X., Hu, X., Poolman, J. T., Crowe, B. A., & Achtman, M. 1992, "Clonal and antigenic analysis of serogroup A Neisseria meningitidis with particular reference to epidemiological features of epidemic meningitis in the People's Republic of China", *Infect.Immun.,* vol. 60, no. 12, pp. 5267-5282

339. Waterboer, T., Sehr, P., & Pawlita, M. 2006, "Suppression of non-specific binding in serological Luminex assays", *J.Immunol.Methods,* vol. 309, no. 1-2, pp. 200-204

340. Watkins, E. R. & Maiden, M. C. 2012, "Persistence of hyperinvasive meningococcal strain types during global spread as recorded in the PubMLST database", *PLoS.One.,* vol. 7, no. 9, pp. e45349

341. Wedege, E., Bjune, G., Froholm, L. O., Hoiby, E. A., & Rosenqvist, E. 1991, "Immunoblotting studies of vaccinee and patient sera from a Norwegian

serogroup B meningococcal vaccination trial", *NIPH Ann.,* vol. 14, no. 2, pp. 183-184

342. Wedege, E., Bolstad, K., Aase, A., Herstad, T. K., McCallum, L., Rosenqvist, E., Oster, P., & Martin, D. 2007, "Functional and specific antibody responses in adult volunteers in new zealand who were given one of two different meningococcal serogroup B outer membrane vesicle vaccines", *Clin.Vaccine Immunol.,* vol. 14, no. 7, pp. 830-838

343. Wedege, E., Bolstad, K., Wetzler, L. M., & Guttormsen, H. 2000, "IgG antibody levels to meningococcal porins in patient sera: comparison of immunoblotting and ELISA measurements", *J.Immunol.Methods,* vol. 244, no. 1-2, pp. 9-15

344. Wedege, E., Caugant, D. A., Musacchio, A., Saunders, N. B., & Zollinger, W. D. 1999, "Redesignation of a purported P1.15 subtype-specific meningococcal monoclonal antibody as a P1.19-specific reagent", *Clin.Diagn.Lab Immunol.,* vol. 6, no. 4, pp. 639-642

345. Wedege, E., Dalseg, R., Caugant, D. A., Poolman, J. T., & Froholm, L. O. 1993, "Expression of an inaccessible P1.7 subtype epitope on meningococcal class 1 proteins", *J.Med.Microbiol.,* vol. 38, no. 1, pp. 23-28

346. Wedege, E. & Froholm, L. O. 1986, "Human antibody response to a group B serotype 2a meningococcal vaccine determined by immunoblotting", *Infect.Immun.,* vol. 51, no. 2, pp. 571-578

347. Wedege, E., Hoiby, E. A., Rosenqvist, E., & Bjune, G. 1998, "Immune responses against major outer membrane antigens of Neisseria meningitidis in vaccinees and controls who contracted meningococcal disease during the Norwegian serogroup B protection trial", *Infect.Immun.,* vol. 66, no. 7, pp. 3223-3231

348. Wedege, E., Hoiby, E. A., Rosenqvist, E., & Froholm, L. O. 1990, "Serotyping and subtyping of Neisseria meningitidis isolates by co-agglutination, dot-blotting and ELISA", *J.Med.Microbiol.,* vol. 31, no. 3, pp. 195-201

349. Wedege, E., Kuipers, B., Bolstad, K., van, D. H., Froholm, L. O., Vermont, C., Caugant, D. A., & van den, D. G. 2003, "Antibody specificities and effect of meningococcal carriage in icelandic teenagers receiving the Norwegian serogroup B outer membrane vesicle vaccine", *Infect.Immun.,* vol. 71, no. 7, pp. 3775-3781

350. Welsch, J. A. & Granoff, D. 2007, "Immunity to Neisseria meningitidis Group B in Adults despite Lack of Serum Bactericidal Antibody", *Clin.Vaccine Immunol.,* vol. 14, no. 12, pp. 1596-1602

351. Welsch, J. A., Ram, S., Koeberling, O., & Granoff, D. M. 2008, "Complement-dependent synergistic bactericidal activity of antibodies against factor H-binding protein, a sparsely distributed meningococcal vaccine antigen", *J.Infect.Dis.,* vol. 197, no. 7, pp. 1053-1061

352. Wenger, J. D., DiFabio, J., Landaverde, J. M., Levine, O. S., & Gaafar, T. 1999, "Introduction of Hib conjugate vaccines in the non-industrialized world: experience in four 'newly adopting' countries", *Vaccine,* vol. 18, no. 7-8, pp. 736-742

353. Weynants, V. E., Feron, C. M., Goraj, K. K., Bos, M. P., Denoel, P. A., Verlant, V. G., Tommassen, J., Peak, I. R., Judd, R. C., Jennings, M. P., & Poolman, J. T. 2007, "Additive and synergistic bactericidal activity of antibodies directed against minor

outer membrane proteins of Neisseria meningitidis", *Infect.Immun.,* vol. 75, no. 11, pp. 5434-5442

354. Wyle, F. A., Artenstein, M. S., Brandt, B. L., Tramont, E. C., Kasper, D. L., Altieri, P. L., Berman, S. L., & Lowenthal, J. P. 1972, "Immunologic response of man to group B meningococcal polysaccharide vaccines", *J.Infect.Dis.,* vol. 126, no. 5, pp. 514-521

355. Yancey, R. J. & Finkelstein, R. A. 1981, "Assmilation of iron by pathogenic Neisseria spp", *Infect.Immun.,* vol. 32, no. 2, pp. 592-599

356. Yazdankhah, S. P. & Caugant, D. A. 2004, "Neisseria meningitidis: an overview of the carriage state", *J.Med.Microbiol.,* vol. 53, no. Pt 9, pp. 821-832

357. Zapata, G. A., Vann, W. F., Rubinstein, Y., & Frasch, C. E. 1992, "Identification of variable region differences in Neisseria meningitidis class 3 protein sequences among five group B serotypes", *Mol.Microbiol.,* vol. 6, no. 23, pp. 3493-3499

358. Zhan, L., Zhang, C. W., Wang, D. L., Xu, X., Meng, L., He, C., Zhou, A. P., & Pei, X. F. 2006, "Construction and expression of Neisseria surface protein (*nspA*) of Neisseria gonorrhoeae in *Escherichia coli*", *Sichuan.Da.Xue.Xue.Bao.Yi.Xue.Ban.,* vol. 37, no. 2, pp. 196-199

359. Zhang, Q., Choo, S., Everard, J., Jennings, R., & Finn, A. 2000, "Mucosal immune responses to meningococcal group C conjugate and group A and C polysaccharide vaccines in adolescents", *Infect.Immun.,* vol. 68, no. 5, pp. 2692-2697

360. Zhao, C., Hellman, L. M., Zhan, X., Bowman, W. S., Whiteheart, S. W., & Fried, M. G. 2010, "Hexahistidine-tag-specific optical probes for analyses of proteins and their interactions", *Anal.Biochem.,* vol. 399, no. 2, pp. 237-245

361. Zhu, P., Morelli, G., & Achtman, M. 1999, "The opcA and (psi)opcB regions in Neisseria: genes, pseudogenes, deletions, insertion elements and DNA islands", *Mol.Microbiol.,* vol. 33, no. 3, pp. 635-650

362. Zollinger, W. D., Dalrymple, J. M., & Artenstein, M. S. 1976, "Analysis of parameters affecting the solid phase radioimmunoassay quantitation of antibody to meningococcal antigens", *J.Immunol.,* vol. 117, no. 5 Pt.2, pp. 1788-1798

363. Zollinger, W. D., Donets, M. A., Schmiel, D. H., Pinto, V. B., Labrie, J. E., III, Moran, E. E., Brandt, B. L., Ionin, B., Marques, R., Wu, M., Chen, P., Stoddard, M. B., & Keiser, P. B. 2010, "Design and evaluation in mice of a broadly protective meningococcal group B native outer membrane vesicle vaccine", *Vaccine,* vol. 28, no. 31, pp. 5057-5067

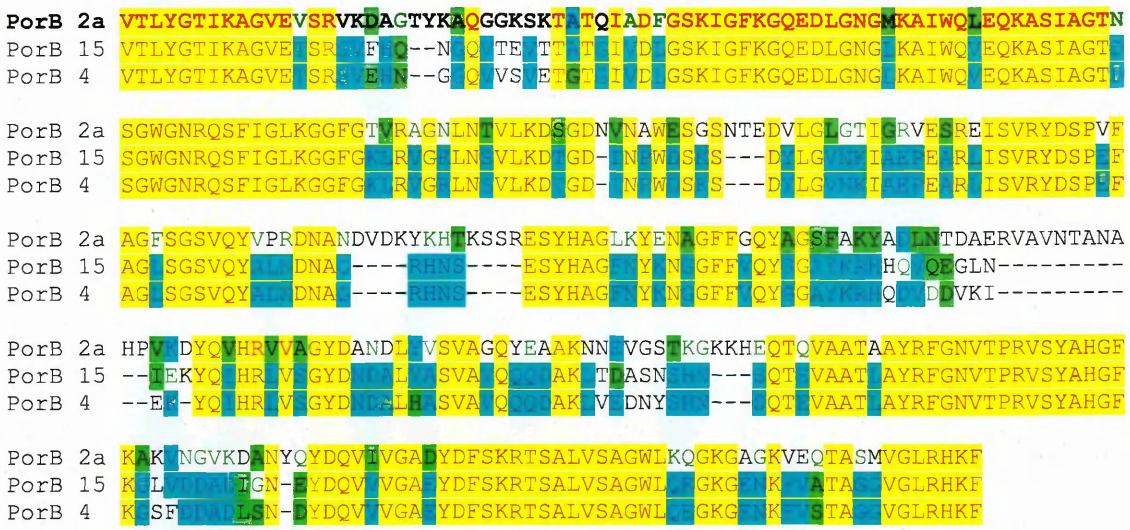
364. Zollinger, W. D., Moran, E. E., & Schmiel, D. H. 2009, "Characterization of an antibody depletion assay for analysis of bactericidal antibody specificity", *Clin.Vaccine Immunol.,* vol. 16, no. 12, pp. 1789-1795

Appendices

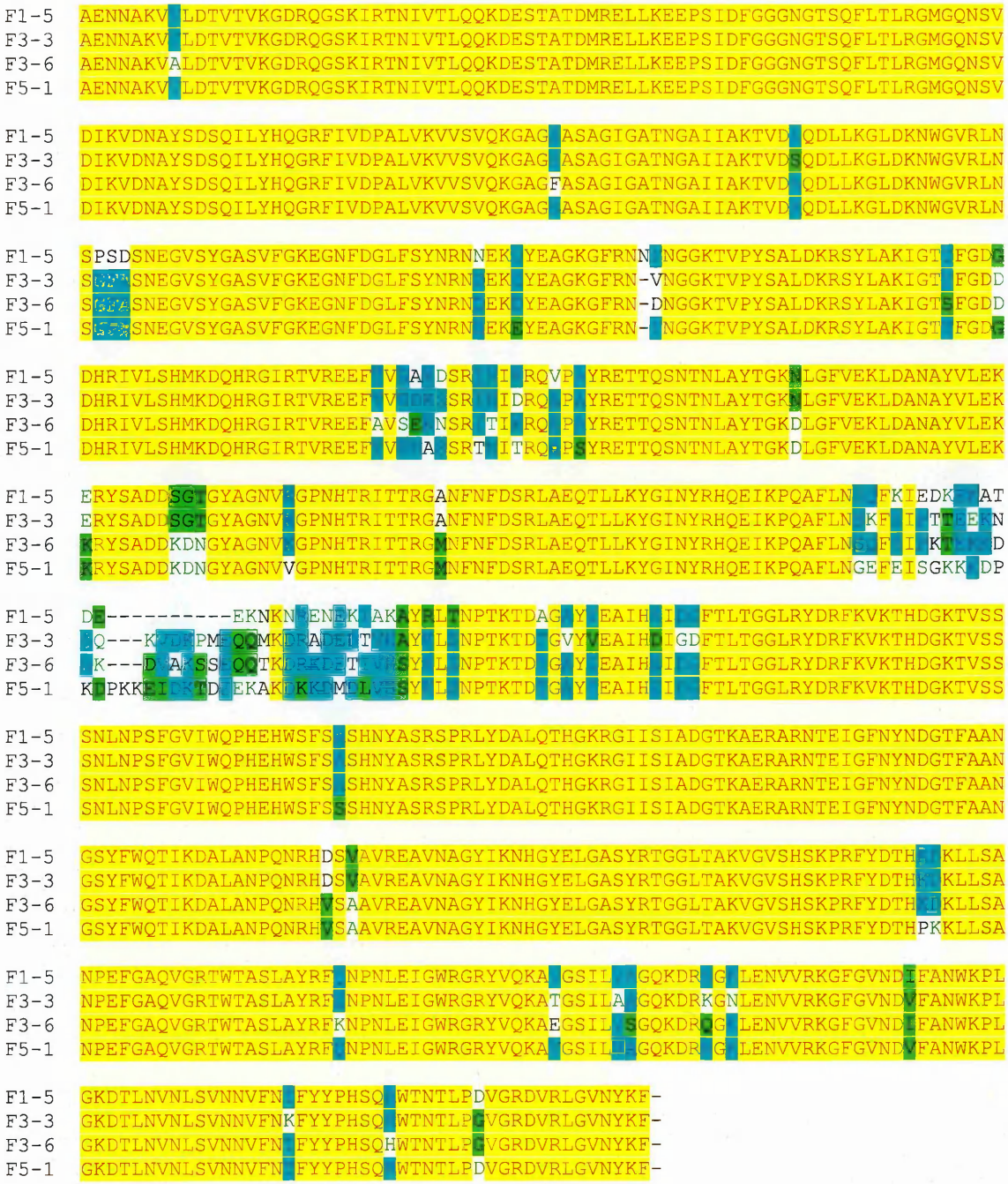
Appendix 1. Alignment of deduced amino acid sequences of the Neisserial OMP genes *porA*, *porB*, *FetA* and fHbp. The alignments were carried out using Vector NTi software.

Alignment of deduced amino acid sequences encoding seven PorA serosubtypes and a VR1 and VR2 deleted mutant (P1._,_)

Alignment of deduced amino acid sequences encoding three subtypes of PorB



Alignment of deduced amino acid sequences encoding four variants of the FetA protein



Alignment of the amino acid sequences of fHbp variants used in this study.